Mechanisms of Pain Processing:
Spinal Protein Translation in the Rat

By

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I, Curtis Oware Asante, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

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Abstract

The word ‘pain’ is described by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. Shifts in pain thresholds and responsiveness are an expression of neural plasticity, a process whereby changes in the nervous system modulate the response to a given stimulus. It is widely believed that this process may contribute to chronic pain. Forms of long-term plasticity specifically require protein synthesis and such mechanisms are widely believed to be cellular counterparts of long-term memory. Relevant to mammalian pain nociceptors is the realisation that messenger ribonucleic acid (mRNA) in dendrites, axons, axon terminals as well as cell bodies, is essential for long-term synaptic plasticity and may therefore be important in pain processing. The protein kinase mammalian target of rapamycin (mTOR) is a key regulator of protein translation and can be specifically inhibited by the drug rapamycin and the rapamycin analogue cell cycle inhibitor (CCI)-779 (CCI-779). This was investigated in rats under physiological conditions and also pathophysiological conditions relevant to clinical pain syndromes i.e. persistent pain-like states. A variety of techniques were utilised: in vivo electrophysiology was used to obtain extracellular single unit recordings of spinal cord lamina V wide dynamic range (WDR) neurones that respond to innocuous and noxious stimuli to peripheral sites i.e. the rat hind paw; behavioural studies were used to assess the progression of pain-like states and assess the effects of rapamycin/CCI-779 on this behaviour and immunohistochemistry was used to visualise active components of rapamycin-sensitive protein translation pathways at the spinal level. In addition, the dependence of these spinal mechanisms on descending serotonergic pathways from higher brain centres was investigated pharmacologically by selectively activating or blocking serotonergic spinal 5-HT3 receptors. These pathways have already been proven to be pivotal in the maintenance of persistent pain-like states. The results suggest that mTOR has a continuous role in maintaining persistent pain-like states via rapid local protein translation, which can be influenced by descending facilitatory controls from higher centres in the brain.

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Abbreviations

ADP	Adenosine diphosphate
Akt	Protein kinase B
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionate
AN	Amygdala nuclei
ANOVA	Analysis of variance
D-AP5	D-2-amino-5-phosphonopentanoate
APP	Amyloid precursor protein
α2AR	Alpha (α) 2 adrenoreceptor
ASIC	Acid sensing ion channel
ATP	Adenosine triphosphate
AUC	Area under the curve
BDNF	Brain-derived neurotrophic factor
CaMKII	Calcium/calmodulin dependent protein kinase II
cAMP	Cyclic adenosine monophosphate
Cav	Voltage dependent calcium channel subtype
CCI	Chronic constriction injury
CCI-779	Cell cycle inhibitor 779
CCL	Chemokine (C-C motif) ligand
CFA	Complete Freund's adjuvant
CGRP	Calcitonin gene related peptide
CNS	Central nervous system
COX	Cyclooxygenase
CREB	cAMP response element binding protein
Cy3	Cyanine 3
DCCP	D-(-)-4-(3-phosphonopropyl)piperazine-2-carboxylic acid
DHPG	(RS)-3,5-dihydroxyphenylglycine

5,7 DHT	5,7-dihydroxytryptamine
DLPT	Dorsolateral pontine tegmentum
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRASIC	Dorsal root acid sensing ion channel
DRG	Dorsal root ganglia
DTA	Diptheria toxin A
4EBP	eIF4E binding protein
eEF1A	Eukaryotic elongation factor 1A
eEF2	Eukaryotic elongation factor 2
EFNS	European Fedaration of Neurological Sciences
eIF4E	Eukaryotic initiation factor 4E
EMG	Electromyography
ERK	Extracellular signal regulated kinase
FITC	Fluorescein isothiocyanate
FKBP12	FK506 binding protein 12
FMRP	Fragile X mental retardation protein
FRAP	Fluoride-resistant acid phosphatase
GABA	Gamma (γ)-amino butyric acid
GAP	GTPase activating protein
GBP	Gabapentin
GDNF	Glial cell-derived neurotrophic factor
GFAP	Glial fibrillary acidic protein
iGluR	Ionotropic glutamate receptor
mGluR	Metabotropic glutamate receptor
GPCR	G-protein-coupled receptor
GTP	Guanine triphosphate

5-HT	5-hydroxytryptamine, also known as serotonin
HN	Hypothalamic nuclei
IASP	International Association for the Study of Pain
IB4	Isolectin B4
eIF4E	Eukaryotic initiation factor 4E
i.c.v.	Intracerebroventricular
IL	Interleukin
i.p.	Intraperitoneal
i.t.	Intrathecal
KO	Knock out
L(n)	Lumbar segment (where n = number of segment e.g. L3)
LLTP	Late phase long term potentiation
LTH	Long term hyperexcitability
LTP	Long term potentiation
MeCP2	Methyl-CpG-binding protein
MOR	Mu (μ)-opioid receptor
MPEP	2-methyl-6-(phenylethynyl) pyridine
MTEP	3[2-methyl-1,3thiazol-4-yl)ethynyl]pyridine
N52	NF200 clone
Nav	Voltage dependent sodium channel subtype
NCF	Nucleus cuneiformis
NF200	200 kDa neurofilament
NGF	Nerve growth factor
NK	Neurokinin
NMDA	N-methyl-D-aspartate
NO	Nitric oxide
nNOS	Neuronal nitric oxide synthase

NR	NMDA receptor subunit
NRMC	Nucleus reticularis magnocellularis
NS	Nociceptive specific
NSAID	Non steroidal anti-inflammatory drug
NT	Neurotrophin
OCT	Optimal cutting temperature compound
P2	Purine receptor
PAG	Periaqueductal gray
PB	Parabrachial
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Post-discharge
PDK1	3-phosphoinositide-dependent protein kinase 1
PDN	Painful diabetic neuropathy
PG	Prostaglandin
PGI2	Prostacyclin
PhB	Phosphate buffer
PHN	Post-herpetic neuralgia
PI3K	Phosphoinositide-3 kinase
PIP3	Phosphatidylinositol 3,4,5-triphosphate
PKA	Protein kinase A
PKC	Protein kinase C
PKCγ	Protein kinase gamma (γ)
PKMζ	Protein kinase M zeta (ζ)
PLP	Phantom limb pain
PN	Parabrachial nuclei
p.o.	Orally

PSL	Partial sciatic nerve ligation
PSTH	Post-stimulus time histogram
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
Rheb	Ras homolog enriched in brain protein
RNA	Ribonucleic acid
mRNA	messenger ribonucleic acid
Runx1	Runt domain transcription factor
RVM	Rostral ventromedial medulla
SAP	Saporin
S(n)	Sacral segment (where n = number of segment e.g. S2)
p70S6K	70 kDa ribosomal protein S6 kinase
SCI	Spinal cord injury
SEM	Standard error of the mean
SNI	Spared nerve injury
SNL	Spinal nerve ligation
SNRI	Serotonin-noradrenaline reuptake inhibitors
SP	Substance P
SAP	Saporin
T(n)	Thoracic segment (where n = number of segment e.g. T13)
TCA	Tricyclic antidepressants
TCTP	Translationally controlled tumour protein
TNFα	Tumour necrosis factor alpha (α)
Trk	Neurotrophin receptor
TMP	Thymidine monophosphate
TRP	Transient receptor potential
TSA	Tyramide signal amplification
TSC1	Hamartin

TSC2	Tuberin
VDCC	Voltage dependent calcium channel
VGluT1	Vesicular glutamate transporter 1
WDR	Wide dynamic range
WT	Wild type

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1 Understanding pain

The word 'pain' is described by the International Association for the Study of Pain (IASP) as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage (IASP, 1994). Nociceptive pain, which is activated by noxious stimuli acting via high threshold sensory pathways, is essential for the survival of organisms in a potentially damaging environment (Millan, 1999). However, although pain is essential, in a chronic form (which outlasts healing, tissue or nerve damage, or continues due to a failure of the injury to heal), it can and often does cause a substantial amount of distress, impacting largely on the patients' quality of life.

Patients in pain are likely to become depressed, anxious and fearful, suffer from disturbed sleep patterns and generally have a poor quality of life, suggesting complex correlations in neuronal signalling within the circuits that underlie pain and its comorbidities. Furthermore, untreated pain has major social and economic impact in terms of lost employment and medical cost. A European survey of chronic pain and its impact on daily life and treatment has revealed just how devastating and widespread chronic pain is. In fact, chronic pain, affects approximately one in five adults in Europe (Breivik et al., 2006). According to the European Pain Network (see http://www.europeanpainnetwork.com), Europeans with chronic pain suffer on average for seven years. The costs to society are huge, with an estimated 500 million lost working days costing € 34 billion a year in Europe. The major clinical chronic pains arise from surgery, trauma and disease and can be grouped as pain from inflammation (rheumatism and arthritis), nerve injury (diabetic neuropathy, AIDS and post-herpetic neuralgia) and cancer. Furthermore, in depth analysis of these chronic pain patients has revealed that 46 % have pain all or most of the time and 34 % of those with chronic pain rate it as severe (Breivik et al., 2006).

The survey by Breivik et al. also revealed that 45 % of chronic pain sufferers had inadequate management of their pain. Only 2 % were treated by a pain specialist and a third of chronic pain sufferers weren't being treated at all. Yet current treatments for chronic pain do exist and include non-medication treatments such as massage, physical therapy and acupuncture; non-prescription analgesics such as

non-steroidal anti-inflammatory drugs (NSAIDs), paracetamol and weak opioids and prescription analgesics such as cyclooxygenase-2 (COX2) inhibitors, strong opioids, antidepressants and anticonvulsants. However, despite recent advances in understanding how pain is processed in the body as well as the wide range of treatments that are currently available, there is still a large unmet clinical need regarding chronic pain syndromes. Furthermore, for many of the drugs listed above, the rationale for prescribing these drugs for chronic pain syndromes is often based on trial and error. However, innovative and integrated studies particularly over the past decade have been pivotal in furthering our understanding of nociception, chronic pain syndromes and appropriate treatments

1.1 Development of nociceptors

Nociceptors (or pain receptors) comprise the large majority of small diameter thinly myelinated Aδ-fibres and unmyelinated C-fibres. Unlike Aδ-fibres, C-fibres are nociceptive specific and have been successfully categorised depending on their physiological properties as well as their anatomical differences. During rodent development, a large proportion of neurones (70 – 80 %) in the cell bodies of the nerve fibres i.e. dorsal root ganglia (DRG) express the nerve growth factor (NGF) receptor tyrosine kinase TrkA during development and require NGF for survival during embryonic life. Most of these neurones also express the Runx1 runt domain transcription factor, calcitonin gene-related peptide (CGRP) and substance P (SP) and project to superficial regions of the spinal cord i.e. lamina I and II (Silos-Santiago et al., 1995; Chen et al., 2006).

During maturation of rodents, the sensitivity of sensory neurones to trophic factors changes such that in the first 3 postnatal weeks of the animal, approximately 50 % of cells lose TrkA meaning that at adulthood, the receptor is only present in about 40 – 50 % of DRG neurones. These cells also express CGRP and SP. The cells that lose TrkA bind isolectin B4 (IB4) and the enzymes fluoride-resistant acid phosphatase (FRAP) and thymidine monophosphate (TMP) and project to the inner layer of lamina II in the dorsal horn. The TrkA-negative neurones also express the receptor tyrosine kinase Ret in the later embryonic stages and also over the first postnatal week and become sensitive to glial cell-derived neurotrophic factor

(GDNF) (Molliver et al., 1997). Other markers of sensory neurones include vanilloid receptor 1 (VR1) which is now more commonly known as transient receptor potential vanilloid 1 (TRPV1). This receptor responds to the chilli extract capsaicin and thermal stimuli (>42 °C) and both IB4-binding and CGRP-expressing neurones respond to capsaicin, indicating that both subsets of nociceptors express TRPV1. In addition, the sensory neurone specific adenosine triphosphate (ATP) receptor P2X3 is highly expressed by IB4-binding, GDNF-sensitive nociceptors and a small minority of peptide containing NGF-sensitive neurones (Snider and McMahon, 1998). To date, there are many studies, which have utilised this information to further categorise nociceptors. For example, transient Runx1 expression is not only required for TRPV1, but also other thermal/chemical receptors including TRPM8 (activated by cold and cooling agents), TRPA1 (activated by chemicals including formalin) and TRPV2 (activated by >52 °C) (Chen et al., 2006). Also, the sensory neurone specific sodium channel Nav1.8 has been shown to be present in all IB4-binding neurones and a small population of CGRP positive neurones (Abrahamsen et al., 2008). Despite this, assigning specific roles for the individual subsets of neurones has remained difficult.

1.2 Peripheral sensory fibre conduction

All sensory information is transmitted from a peripheral site where the stimulation originates e.g. the skin, along primary afferent fibres including nociceptors to the spinal cord where the signal is transferred via neurotransmitter release from the central terminals to the spinal neurones in the cord. As would be expected, due to the wide range of peripheral stimuli to which humans and animals are sensitive, sensory information is transmitted via nerves, which are specific for particular modalities. These nerve fibres are named Aβ, Aδ and C, each with their own specific features that contribute to enabling sensation of innocuous and noxious stimuli.

Aβ-fibres, or low threshold mechanoreceptors transmit innocuous mechanical stimuli as well as tissue displacement, velocity of displacement and vibration placement and are in close contact with tissue supporting structures such as epidermal cells and connective tissue. These fibres are of large diameter and are

heavily myelinated, allowing rapid conduction of action potentials to the spinal cord. Aδ-fibres are smaller in diameter than Aβ-fibres and are lightly myelinated therefore making them slower conducting than Aβ-fibres and these fibres respond to mechanical and noxious thermal stimuli (Myers, 1997; Julius and Basbaum, 2001; D'Mello and Dickenson, 2008). Although Aδ- and C-fibres are classified as nociceptors, the light myelination of Aδ-fibres means that their conduction velocity overlaps slightly with Aβ-fibres. Aβ-fibres conduct at a speed of 30 – 80 m/s; Aδ-fibres conduct at a velocity of 6 – 30 m/s and C-fibres conduct at a velocity of 0.5 – 2 m/s (Schmidt and Willis, 2007). The slight overlap in conduction velocities of Aβ- and Aδ- fibres explains why some Aδ-fibres display Aβ-fibre properties and vice versa. C-fibres however are unmyelinated and of the smallest diameter and therefore possess the slowest conduction velocities. C-fibres are generally thought of as being nociceptive specific and many C-fibres are polymodal in their response to noxious mechanical and thermal stimulation as well as chemical stimulation (D'Mello and Dickenson, 2008).

1.3 Peripheral sensitisation

Peripheral sensitisation refers to a form of stimulus evoked functional plasticity of nociceptors at peripheral sites (i.e. before the dorsal root entry zone) whereby due to injury or tissue damage, inflammatory mediators sensitise the nociceptors to the extent where the threshold is reduced and responsiveness to further stimuli is enhanced. More specifically, injury or inflammation can result in the upregulation of a large number of sensitising agents that cause peripheral sensitisation as a result of cell disruption, mast cell degranulation and inflammatory cell secretion (Woolf and Ma, 2007). This list includes NGF, bradykinin, serotonin, ATP, protons (H^+), histamine, prostaglandins and lipids (Julius and Basbaum, 2001) as well as cytokines and nitric oxide (NO) (Marchand et al., 2005). More recently, other key players have also been identified including the transforming growth factor β (TGFβ) member activin, tumour necrosis factor α (TNFα), chemokine (C-C motif) ligand 3 (CCL3), GDNF and proteases such as thrombin and trypsin (Woolf and Ma, 2007). These mediators will directly or indirectly modulate various receptors that are located on peripheral terminals and/or axons of nociceptors, most of which are summarised in tables 1.1 and 1.2.

An example of a receptor that has received much attention in the past decade, particularly in relation to peripheral sensitisation is TRPV1. This receptor is located on nociceptors in the peripeheral nervous system as well as spinal neurones within the central nervous system (CNS). Some pro-inflammatory mediators including protons and lipid mediators e.g. anandamide can directly activate TRPV1. In addition, TRPV1 can also be modulated by the action of protein kinases, phosphatases and/or lipid messengers produced by receptor-coupled intracellular signalling pathways. Some inflammatory mediators (e.g. bradykinin) bind to G-protein-coupled receptors (GPCRs) which signal via PKA and PKC to sensitise TRPV1. In addition, some inflammatory mediators including prostaglandins, bradykinin and NGF may induce hyperalgesia by increasing the expression of TRPV1 at the cell surface (Huang et al., 2006).

Receptor/channel	Major subtypes	Ligands	Notes
Purine	P2X2, P2X3	ATP, ADP, adenosine	P2X2 is modulated by protons; P2X3 is GDNF regulated
Acid-sensing proton-gated	DRASIC, ASIC (α, β)	Protons	Amiloride-sensitive, Mechanosensitive?
Vanilloid	TRPV1	Heat, capsaicin	Sensitised by heat; proton-gated; NGF regulated
Sodium tetrodotoxin resistant	Nav1.8, Nav1.9	N/A	Protein kinase A/C (PKA/C) substrate; NGF regulated
Sodium tetrodotoxin sensitive	Nav1.6, Nav1.7	N/A	NGF regulated
Voltage-dependent calcium channels (VDCCs)	T- (low threshold), L- and N-type (high threshold)	N/A	Secondary calcium currents mediated by GPCRs
Serotonin	5-HT3	Serotonin	
NMDA	NR1/2 heterotetramers	Glutamate/ aspartate	Controls SP release
AMPA	iGluR 1-3	Glutamate	
Kainate	iGluR5	Glutamate	Controls neurotransmitter release

Table 1.1 Common Ionotropic receptors and ligand gated ion channels expressed by nociceptive primary afferent neurones that are implicated in persistent and chronic pain-like states (adapted from Woolf and Costigan, 1999).

Receptor/Channel	Major subtypes	Ligands	Notes
Prostanoids Prostaglandins	EP1 - 4	PGE1 - 4	PGE2 sensitises some normally unresponsive cells to bradykinin; PGE2-PKA/C mediated modulation of Nav1.8 and 1.9; sensitises heat stimuli
Prostacyclin	IP	PGI2	
Histamine	H1	Histamine	Itch mediator
Serotonin	5-HT1A, 5-HT2A, 5-HT3, 5-HT4	Serotonin	5-HT4 increases Nav1.8 and 1.9 currents via PKA/C
Bradykinin	B1 (induced), B2	Bradykinin	B1 sensitises cells to heat via PKA B2 increases the number of cells that respond to capsaicin and protons
Cannabinoid	CB1 - 2	Anandamide	Secondary calcium currents mediated by GPCRs
Tachykinin	NK1	SP, neurokinin A	
Opioid	μ, δ, κ	Enkephalins, dynorphins, β-endorphins	Inflammatory cells release endogenous opioids. Inhibits peripheral activation

Table 1.2 Common metabotropic receptors expressed by nociceptive primary afferent neurones that are implicated in persistent and chronic pain-like states (adapted from Woolf and Costigan, 1999).

1.4 Organisation of the spinal cord

All sensory afferents terminate at specific locations in the spinal cord, a process that is highly organised and dependent on not just the nature of the nerve fibre, but also the environment into which they terminate. The point at which the nerve fibres enter the region of the spinal cord is called the dorsal root entry zone. In the early 1950's Rexed reported on his organisation of the spinal cord into a series of layers that shared the same features from the sacral to the cervical cord with minor variations (Rexed, 1952). This organisation involved dividing the grey matter into specific laminae numbered from I to X, whereby lamina I to VI form the dorsal horn of the spinal cord and laminae VII to IX the ventral horn. Lamina X is the substantia grisea centralis i.e. the grey matter surrounding the central canal (see figure 1.1). Below, is more detail regarding lamina I, II and V, which are of most relevance to this thesis.

1.4.1 Lamina I

Lamina I is the most superficial lamina of the dorsal horn of the spinal cord and receives input from small diameter unmyelinated C-fibres and finely myelinated Aδ-fibres. This is the termination region for CGRP/TrkA-expressing nociceptors as well as SP/TrkA-expressing nociceptors (they also terminate in the outer region of lamina II) (Snider and McMahon, 1998). Lamina I consists of small neurones that are mainly nociceptive specific (NS) in nature and some are thermoreceptive or sensitive to itch stimuli. Lamina I neurones project to the brain via long-distance tracts such as the ascending spino-parabrachial tract and other neurones project laterally to other neurones within the same lamina or dorsally to other neurones in deeper laminae. Lamina I neurones are therefore able to modulate the excitability of deeper laminae neurones directly from connecting pathways within the spinal cord or via relay systems which feed back down to spinal cord via descending tracts (Sorkin and Carlton, 1997; Suzuki et al., 2002; Dickenson and Bee, 2008).

1.4.2 Lamina II

Lamina II or the substantia gelatinosa receives input from CGRP/TrkA-expressing DRG nociceptors to the outer layer and IB4-binding and Ret-expressing DRG nociceptors to the inner layer, which is also the termination zone for Aδ-fibres. The neurones of lamina II form a layer of interneurones that, as well as forming lateral connections with other interneurones in lamina II, also project to other neurones in laminae above and below it, although they don't appear to form connections with supraspinal sites (Sorkin and Carlton, 1997; Snider and McMahon, 1998; Dickenson and Bee, 2008). Of particular interest is the fact that the neuronal specific γ form of protein kinase C (PKCγ) is specifically expressed throughout inner lamina II in the target field of Ret-expressing neurones but not in primary sensory neurones themselves. Studies involving a transgenic line of mice lacking PKCγ revealed that although behavioural responses to thermal and mechanical stimuli were normal, hypersensitivity induced by nerve injury was significantly reduced (Malmberg et al., 1997; Snider and McMahon, 1998). The majority of the PKCγ interneurones have been found not to be γ-amino butyric acid (GABA)- immunoreactive or immunoreactive for the μ-opioid receptor 1 (MOR-1) but instead immunoreactive for neurotensin and somatostatin, indicating a restricted expression profile to specific excitatory neurones (Polgar et al., 1999).

1.4.3 Lamina V

Lamina III and IV are known collectively as the nucleus proprius and contain cells that respond to innocuous input such as that arriving along Aβ-fibres (Dickenson and Bee, 2008). Deeper down in the spinal cord at lamina V resides a population of neurones that are also important in pain perception and therefore chronic pain states. These neurones are known as wide dynamic range (WDR) neurones (and are also found in lamina VI), so called due to their ability to respond to both innocuous and noxious stimuli. They also receive input directly or indirectly from all sensory afferent subtypes (see figure 1.1.) WDR neurones typically respond to stimuli applied to large receptive fields and can 'wind up', a term describing the temporal summation of the responses of the neurone to repeated stimuli of the same

intensity (Mendell and Wall, 1965) and this is mediated by C-fibre afferents (Mendell, 1966).

1.4.4 Mechanism of wind up

Wind up is largely dependent on the excitatory neurotransmitter glutamate. Glutamate exerts excitatory effects throughout the nervous system, so it is therefore not surprising that it should have important roles in pain processing and it has been shown to act at postsynaptic sites on spinal neurones containing the glutamatergic α-amino-3-hydroxy-5-methyl-4-isoxazeloproprionic acid receptors (AMPARs); N-methyl-D-aspartate receptors (NMDARs) and G-protein coupled metabotropic glutamate receptors (mGluRs) as well as pre-synaptic kainite receptors (D'Mello and Dickenson, 2008).

The amount and frequency of glutamate release is dependent on sensory afferent stimulation such that the more noxious a stimulus is, the more glutamate that is released. Fast acting AMPARs are thought to be the initial responders to glutamate release. When there is an increase in C-fibre activity due to e.g. noxious stimuli, prolonged glutamate release now results in wind up due to the recruitment of initially silent NMDARs (Dickenson and Sullivan, 1987). This silence is due to a physiological block of the receptor ion channel pore by magnesium ions (Mg^{2+}). Sustained depolarisation of the spinal neurones mediated at least in part by AMPA receptors results in removal of the Mg^{2+} block thus allowing entry Ca^{2+} into spinal neurones. In accordance with this, AMPA receptor antagonists have been shown not to be effective in altering wind up (Stanfa and Dickenson, 1999; Seagrove et al., 2004). Furthermore, release of other transmitters from C-fibres such as SP and CGRP are likely to contribute to the sustained depolarisation of spinal neurones and thus removal of the Mg^{2+} block which would lead to wind up (Budai et al., 1995; Khasabov et al., 2002; Suzuki et al., 2003).

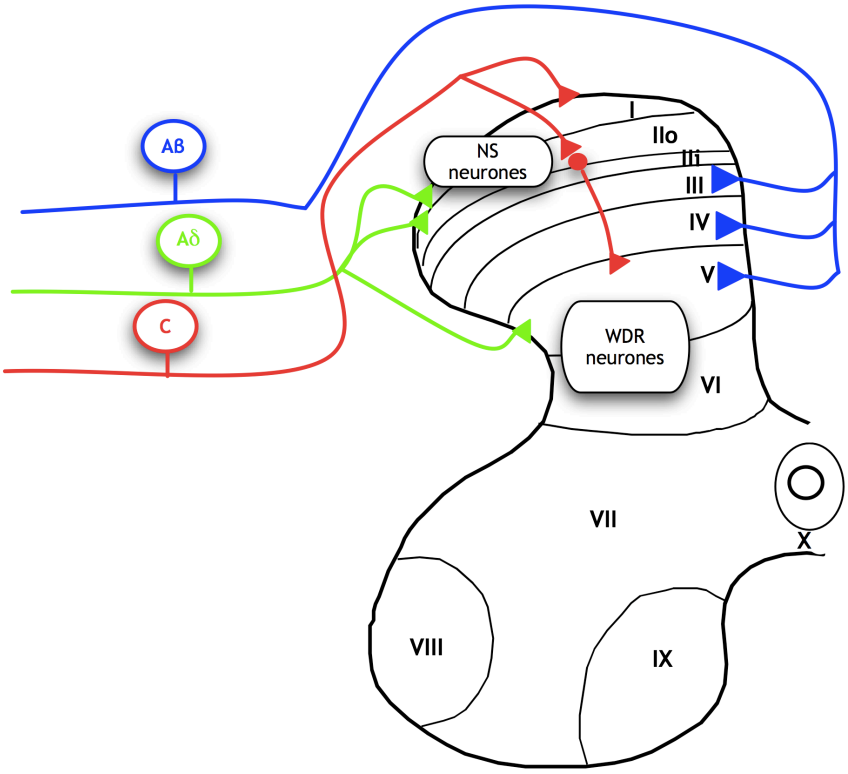


Figure 1.1 Sensory pathways from the periphery to the spinal cord. Primary afferent fibres comprising Aβ(blue)-, Aδ(green)- and C-fibres (red) transmit signals from the periphery to the spinal cord. NS neurones are restricted to the superficial laminae of the dorsal horn (laminae I and II) and receive input from nociceptors i.e. Aδ- and C-fibres. WDR neurones are located deep in Lamina V (and VI) and receive input directly or indirectly from all sensory neurone subtypes (adapted from D'Mello and Dickenson, 2008).

1.5 Ascending projections from the spinal cord to the brain

After signals are transmitted to the spinal cord, where they are sorted and processed, they are then sent to the brain via spinal projection neurones where they are analysed and transformed into 'painful sensations'.

1.5.1 Lamina I projections

A major population of projection neurones reside in lamina I, of which most (80 %) can be identified by immunoreactivity for the neurokinin 1 (NK1) receptor for SP (Todd, 2002). In terms of pain processing, the main projection pathway is the dense spinoparabrachial pathway. In this pathway, the parabrachial (PB) nuclei collect and process signals from spinal neurones before they are transmitted to higher brain centres (Blomqvist et al., 1989; Bester et al., 1997). These areas include the amygdala, hypothalamus and the periaqueductal grey (PAG). The amygdala is involved in mediating aversion, anxiety and fear-induced avoidance learning (Maren, 2007), the hypothalamus mediates homeostatic processes such as blood pressure and heart rate (Vidal et al., 1984) and the PAG mediates opioid and non-opioid endogenous analgesia (Lewis and Gebhart, 1977). Another ascending pathway that connects lamina I to the brain, albeit less dense than the spinoparabrachial pathway, is the spinothalamic pathway, whereby projection neurones project to caudal regions of thalamus. The thalamus then projects to the insular and somatosensory cortices, thus enabling sensory discrimination (Bester et al., 2000). It should be noted that there are also a number of projection neurones that are found in lamina III and IV that project predominantly to the thalamus, thus contributing to the spinothalamic tract (D'Mello and Dickenson, 2008).

1.5.2 Lamina V projections

Also contributing to the spinothalamic tract are projection pathways, which connect spinal neurones in even deeper laminae with the thalamus (Lima et al., 1991; Hunt and Mantyh, 2001). The thalamus sends projections onto the prefrontal cortex, which has been shown to be involved in attention and the motivational aspects of pain (Wiech et al., 2005).

1.6 Descending projections from the brain to the spinal cord

The 1970s was a period that witnessed a flurry of research into descending systems and their modulation of nociception. Pivotal to this was a study, which reported that focal electrical stimulation in the rat midbrain PAG allowed abdominal surgery (laparotomy) to be carried out on animals without general anaesthesia. This work (Reynolds, 1969) acted as a catalyst not only in identifying the PAG and adjacent areas as modulators of analgesia in awake animals, but also into the research which has contributed to the discovery of the dynamic features relevant to descending systems. The descending systems described below form part of the spino-bulbo-spinal loop i.e. the circuitry that comprises ascending connections from the spinal cord to the brain and descending connections from the brain back to the spinal cord where incoming signals from the periphery as well as neuronal processing within the cord are further modulated (see figure 1.2)

1.6.1 The PAG

Not long after the work of Reynolds, was the discovery by Hertz et al. who showed that injecting morphine directly into the PAG area resulted in analgesia from a nociceptive stimulus (Herz et al., 1970). This study, in addition to the identification of specific opioid binding sites in the brain (Pert and Snyder, 1973) and the isolation of endogenous opioid-like peptides in the brain (Hughes, 1975) confirmed that not only was the PAG necessary for inhibition of spinal nociceptive processing, but that its inhibitory action was mediated by endogenous opioid-like peptides such as enkephalins. Also of major importance during this era was the finding that stimulus-induced analgesia of the PAG could be achieved if it was administered for several minutes prior to the onset of a painful stimulus during which, specific neurones are recruited or neurotransmitters released that act either at the origin of the stimulus or at sites further away such as the spinal cord (Melzack and Melinkoff, 1974).

Descending neurones from the PAG terminate in the dorsolateral pontine tegmentum (DLPT), another site that is rostral to the RVM. Within this structure, the locus coeruleus, the A5 and A7 noradrenergic cell groups are the main source

of noradrenergic projections to the dorsal horn and are implicated in inhibiting nociception (Proudfit and Clark, 1991; Millan, 2002; Rahman et al., 2008). At the level of the spinal cord, noradrenaline acts predominantly at the $\alpha 2$ adrenoreceptor ($\alpha 2$AR) subclass, thus inhibiting transmitter release from primary afferent terminals, which suppresses firing of projection neurones in the dorsal horn (Millan, 2002; Rahman et al., 2008).

1.6.2 The rostroventral medulla

Although the PAG is clearly of major importance, it is the rostroventral medulla (RVM) that provides the common output for descending influences from rostral sites such as the PAG (Gebhart, 2004).

The RVM contains the nucleus raphae magnus as well as the adjacent reticular formation, including the nucleus reticularis magnocellularis (NRMC) (Mason, 2001). Interestingly, stimulation of the RVM has been reported to both facilitate and inhibit nociceptive responses (Suzuki et al., 2004a). In accordance with these findings, cells within the RVM have been classified into three distinct groups based on specific evoked responses to noxious heat stimulation. “Off” cells are indirectly excited by opioids and inhibited by nociceptive input such that just before a nociceptive reflex, they will display a transient interruption in their discharge. “On” cells are inhibited by opioids and excited by nociceptive input such that just before a nociceptive reflex, they will display a transient increase in their discharge (Millan, 2002). “Neutral” cells maintain their discharge rate irrespective of a nociceptive reflex, although their role in pain processing is still a source of debate (Gao and Mason, 2000).

It is now widely accepted that the RVM can modulate neuronal activity at the spinal level via descending influences that exert opposing effects. This is attributable to the fact that descending pathways from the RVM modulate neuronal activity by releasing a range of neurotransmitters including serotonin (5-hydroxytryptamine or 5-HT), GABA, acetylcholine and enkephalins. These transmitters can act at either multiple subtypes of receptors differentially coupled to intracellular signalling mechanisms or a single receptor subtype localised on

different classes of target spinal neurones (Millan, 2002). Of these neurotransmitters, 5-HT has received a huge amount of interest. There are more than 15 5-HT receptor (5-HTR) subtypes so it is therefore not surprising that this neurotransmitter is implicated in both anti-nociceptive and pro-nociceptive effects depending on the receptor subtype it acts on. Importantly, although descending facilitation occurs along with descending inhibition, it is the shift in balance towards facilitation that is proposed to be key in contributing to persistent pain states. There are many studies that have focussed on the role of spinal 5-HT3Rs in nociception and persistent pain-like states (Green et al., 2000; Suzuki et al., 2002; Zeitz et al., 2002; Suzuki et al., 2004b; Suzuki et al., 2005; Donovan-Rodriguez et al., 2006; Svensson et al., 2006). Like other spinal 5-HTRs, these receptors are located on primary afferent terminals in the dorsal horn as well as inhibitory interneurones containing enkephalins and GABA (Millan, 2002; Conte et al., 2005). However, unlike all the other 5-HTRs, 5-HT3Rs are the only ligand gated ion channel (Barnes and Sharp, 1999; Millan, 2002).

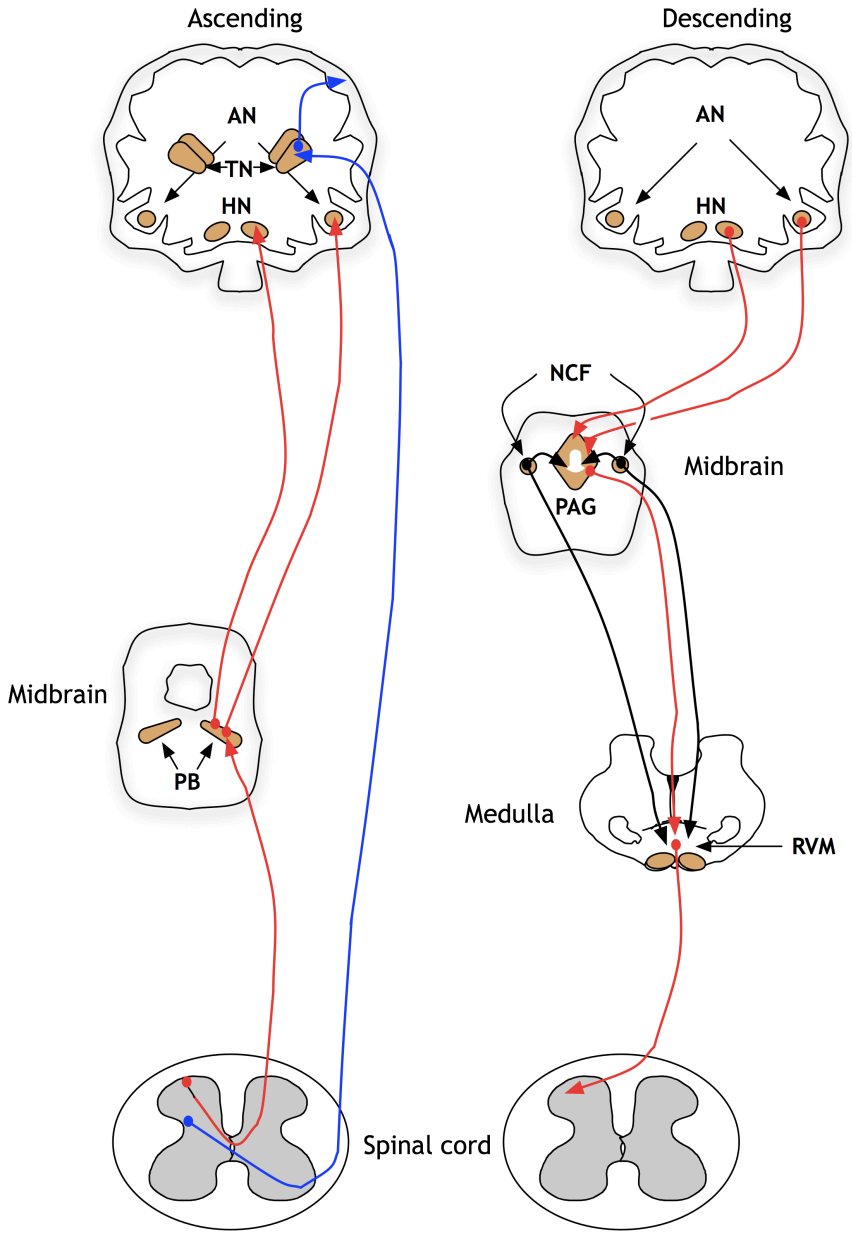


Figure 1.2 Ascending and descending components of the spino-bulbo-spinal loop. (Left) Ascending component shows projections from lamina I neurones in the dorsal horn of the spinal cord (red) and lamina V (blue) to midbrain and higher brain centres. (Right) Descending component shows projections from higher, midbrain and brainstem regions to the dorsal horn of the spinal cord. AN, amygdala nuclei; HN, hypothalamic nuclei; NCF, nucleus cuneiformis; PAG, periaqeductal grey, PB, parabrachial nuclei; TN, thalamic nuclei; RVM, rostroventral medulla (adapted from Hunt and Mantyh, 2001; Tracey and Mantyh, 2007).

1.7 Central sensitisation

Clinically, peripheral sensitisation can result in allodynia whereby non-painful stimuli become painful and hyperalgesia whereby painful stimuli become even more painful. The afferent barrage into the spinal cord often results in central sensitisation whereby neurones in the spinal cord and those in the brain centres associated with pain now also become more responsive to incoming signals, thus further potentiating signals that were originally too weak to initiate a response, resulting in wide spread pain beyond the site of injury (Suzuki et al., 2004b; Suzuki et al., 2005; Basbaum et al., 2008). This can be clearly demonstrated by inducing wind up or short-term potentiation of spinal neurons, a method that can be successfully employed as a read-out of central sensitisation (see section 1.4.4).

1.7.1 Central primary afferent terminals

From figure 1.1, it can be seen that the central terminals of primary afferent sensory fibres terminate in the dorsal horn of the spinal cord. Although glutamate is the main excitatory neurotransmitter in the CNS, nociceptors also transmit signals via the release of neuropeptides such as CGRP and SP and proteins such as brain-derived nerve growth factor (BDNF). Transmitter release has long been known to be dependent upon Ca^{2+} influx after the arrival of action potentials along the axon and to the terminals. The main voltage-dependent calcium channel (VDCC) expressed by nociceptors is Cav2.2 (Castiglioni et al., 2006). Interestingly, the $\alpha 2\delta$ accessory subunit of VDCCs has been identified as the binding site for the drug gabapentin (GBP), which has efficacy in neuropathic pain (Field et al., 2006). Furthermore, the $\alpha 2\delta$ accessory subunit has been shown to be upregulated in central nociceptor afferent terminals after peripheral nerve injury, therefore representing a specific target for the treatment of chronic pain syndromes (Luo et al., 2001).

Transmitter release from central afferent terminals can either be inhibited or potentiated via endogenous mechanisms, which act to modulate this process. For example, transmitter release can be increased by prostaglandin E2 (PGE2), which is produced by COX2 in dorsal horn neurones in response to inflammation.

Transmitter release can also be increased by bradykinin, which increases Ca^{2+} influx through TRPA1 channels. As well as facilitating transmitter release, there are also endogenous mechanisms that act to inhibit transmitter release from nociceptive afferents. These include endogenous opioids acting on μ- and δ-opioid receptors, GABA acting on GABAB receptors and endogenous cannabinoids acting on CB1 receptors. These mechanisms also come into play during persistent pain states, yet in persistent pain states, the facilitatory mechanisms outweigh the inhibitory mechanisms (Woolf and Ma, 2007).

1.7.2 Dorsal horn neurones

The sustained afferent input to the spinal cord from the central terminals of the primary afferents due to injury/inflammation results in an increased sensitivity of the spinal neurones to incoming afferent input. Thus a state of spinal sensitisation occurs resulting in an increase in excitability of dorsal horn units such that responses to peripheral stimuli are enhanced (Chapman et al., 1998; Suzuki et al., 2000).

At the cellular level, central sensitisation is mediated by changes that act to increase spinal neuronal activity. As already discussed, wind up can be used as a read-out of central sensitisation (i.e. wind up increases with central sensitisation) and this means that NMDAR-dependent mechanisms are in part responsible for the neuronal hyperexcitability that results from central sensitisation (Haley et al., 1990; Chapman et al., 1994). Wind up and therefore central sensitisation are not only dependent upon incoming signals from peripheral afferents. Central sensitisation at the spinal level is also modulated by descending signals from higher brain regions.

Innovative studies have revealed key features about the interactions between descending facilitatory pathways from the brain to the spinal cord. Behavioural studies have revealed that injections of the anaesthetic agent lidocaine into the RVM can reverse behavioural hypersensitivity in nerve-injured rats (Burgess et al., 2002). Following on from these studies, in vivo electrophysiology has been used to study spinal neurones after RVM lidocaine treatment (Bee and Dickenson, 2007).

This study showed that by injecting lidocaine into the RVM, dorsal horn WDR neuronal responses to peripheral stimuli could be reduced. This effect was even greater in nerve-injured rats that were hypersensitive to previously innocuous stimuli, suggesting a mechanism for clinical allodynia.

At the root of descending modulation by the RVM are the group of projection neurones in the dorsal horn that express NK1. It has been shown that by ablating lamina I/III NK1-expressing neurones with SP conjugated to the toxin saporin (SP-SAP), that the excitability of WDR neurones could be reduced. In addition, spontaneous neuronal activity produced by injecting the inflammatory agent formalin into the hind paw is reduced in SP-SAP rats, as is the persistent behavioural hypersensitivity that occurs after the inflammatory agent complete Freund's adjuvant (CFA) is injected into the hind paw (Suzuki et al., 2002). These descending modulatory actions were determined to be via the action of serotonin on 5-HT3Rs due to the fact that spinally administering the selective 5-HT3R antagonist ondansetron produced the same inhibitions of mechanically and thermally evoked stimuli as those produced by SP-SAP (Suzuki et al., 2001). In addition, ondansetron has been shown to be more effective in attenuating spinal neuronal responses to peripheral mechanical and thermal stimuli in nerve-injured rats compared to sham controls, suggesting that in persistent pain pain-like states, there is an increase in the facilitatory serotonergic component of descending pathways from the RVM (Suzuki et al., 2004b).

SP-SAP treatment has also been shown to attenuate behavioural hypersensitivity and stimulus evoked neuronal responses in nerve-injured rats (Suzuki et al., 2005). As well as reducing mechanical and thermal stimuli, SP-SAP treatment also blocks wind up and a more sustained form of wind up, long-term potentiation (LTP). This feature however is not mimicked by administration of spinal ondansetron (Rygh et al., 2006). This indicates that some mechanisms of spinal sensitisation do not require descending facilitatory pathways to be active, yet they can be modulated by these pathways.

Descending facilitatory pathways from the RVM are not only important for the behavioural hypersensitivity observed in persistent pain-like states and the spinal neuronal activity that is the basis for this behaviour, but they are also necessary for state-dependent drugs to exert their effects. Such is the case with GBP. In vivo electrophysiology studies have shown that spinal SP-SAP can actually block the inhibitory effects of systemically administered GBP in nerve-injured rats (Suzuki et al., 2005). In addition, selectively blocking the 5-HT3R with ondansetron also prevents the inhibitory actions of GBP. Interestingly, selective activation of 5-HT3Rs with the agonist 2-methyl 5-HT, a method employed to mimic persistent pain-like states, renders GBP effective in naive rats.

Although descending facilitatory action at 5-HT3Rs would appear to be a feature of all persistent pain-like states, this is not the case. The well-established carrageenan-induced inflammation model, whereby the seaweed extract carrageenan is injected into the hind paw is one such example of this. This model presents with behavioural, mechanical and thermal hypersensitivity as well as changes in neuronal plasticity (Kayser and Guilbaud, 1987; Stanfa et al., 1992). As described above, ondansetron will attenuate spontaneous neuronal hyperexcitability due to formalin-induced inflammation as well as mechanically and thermally evoked neuronal responses to a greater degree in nerve-injured rats compared to naive and sham rats. However, in the carrageenan-induced inflammation model, ondanstetron will attenuate neuronal responses to the same extent as naive rats (Rahman et al., 2004) meaning that descending pathways acting at spinal 5-HT3Rs are unaltered. Figure 1.3 shows an overview of many of the components involved in peripheral and central sensitisation.

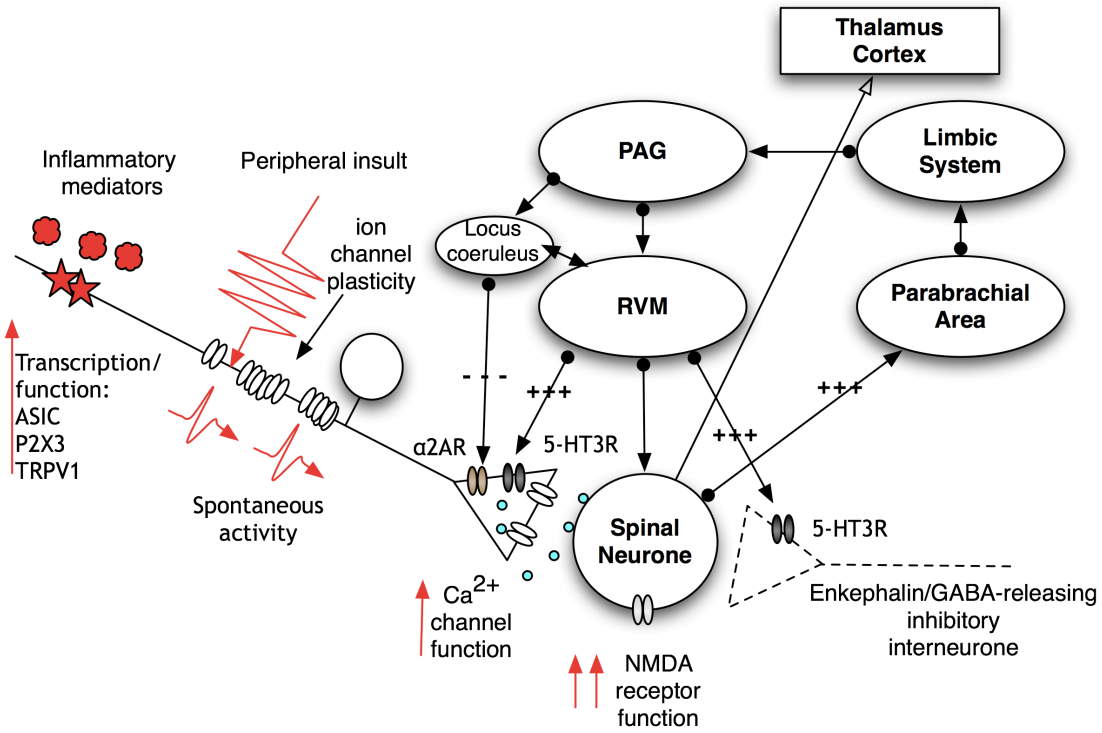


Figure 1.3 Overview of the spino-bulbo-spinal loop and components of peripheral and central sensitisation. Nociceptive signals are transmitted from the periphery to the spinal cord via nociceptive fibres. Peripheral sensitisation as a result of inflammation due to inflammatory mediators or direct nerve injury contributes to hypersensitivity (clinical allodynia and hyperalgesia) and can occur via the upregulation of transcription factors for ion channels that contribute to spontaneous activity. Sustained peripheral sensitisation often results in central sensitisation whereby neurones of the spinal cord and brain regions act to further enhance pain perception. Specifically, central sensitisation can be demonstrated through an increased amount of wind up in spinal neurones. Wind up is an NMDAR-dependent process mediated by C-fibres that involves increased neuronal output to suprathreshold stimuli. Signals from spinal neurones are processed and transmitted rostrally to the parabrachial area in the brainstem, which is associated with the affective aspects of pain. These signals are then relayed back to the spinal cord via the PAG and RVM. During many persistent pain-like states, there is an increase in descending serotonergic modulation acting at 5-HT3Rs on primary afferent terminals that overrides descending inhibitory modulation acting at e.g. α2ARs as well as facilitation of inhibitory interneurones (adapted from Harvey and Dickenson, 2008).

1.8 Peripheral and central sensitisation: a role for non-neuronal cells

The neuronal system is not the only cellular system that is involved in nociception and the changes associated with persistent pain-like states. Recently, there has been a surge in research that seeks to delineate the roles that inflammatory mediators from immune cells play in persistent pain states. It is now clear that these cells and their mediators have significant roles in inflammatory and neuropathic pain-like states (Scholz and Woolf, 2007). In the periphery, Schwann cells, mast cells, macrophages and T-cells are recruited to the site of injury and will release factors such as tumour necrosis factor α (TNFα), interleukin 1b, (IL1b), IL6, CCL2, prostaglandins and NGF that contribute to the initiation and maintenance of sensory abnormalities post-injury. Centrally, primary afferent terminals are surrounded by microglia. These cells are believed to be important in maintaining the physiological conditions of the spinal cord. In persistent pain states such as neuropathy, aberrant transmitter release from primary afferent terminals activates microglia, resulting in a release of TNFα, IL1b, IL6, nitric oxide (NO), ATP and prostaglandins which lead to neuronal hyperexcitability (Marchand et al., 2005).

1.9 Central sensitisation and intracellular signalling pathways

Despite the progress that has been achieved in determining the transmitters and receptors involved in nociception and pain maintenance, our understanding of the intracellular signalling mechanisms regulating nociceptor sensitisation downstream of ligand binding to receptors is still at an early stage (Hucho and Levine, 2007). However, individual signalling pathways are often activated by more than one ligand/receptor combination. These pathways, especially in persistent pain-like states act to promote mechanisms of spinal plasticity such as the more sustained form of wind up, LTP.

1.9.1 LTP as a read-out of central sensitisation

LTP has been studied as a cellular model of synaptic plasticity for many years and is generally defined as a long-lasting, but not necessarily irreversible increase in synaptic plasticity (Sandkuhler, 2007). Early phase LTP is independent of de novo protein synthesis and lasts for up to 3 hr whereas late phase LTP (LLTP) involves protein synthesis and lasts longer than 3 hr, up to the lifespan of the animal and involves structural changes at the synapse (Bailey et al., 2004). LTP was classically demonstrated at synapses in the hippocampus (Bliss and Lomo, 1973) and is still used as a model for learning and memory formation.

More than 100 molecules have been implicated as mediators of hippocampal LTP and many of these are also involved in central sensitisation observed in persistent pain-like states (Ji et al., 2003). This means that hippocampal LTP studies can often be used to predict what transmitters/receptors/signalling cascades are important in central sensitisation and therefore pain maintenance. One prime example of this is the identification of the immediate early gene zif268 as an important determinant of hippocampal LTP. As well as being important for hippocampal LTP, this gene has also been shown to be important for the behavioural outcome of hippocampal LTP i.e. learning and memory. Interestingly, it has been found that spinal LTP results in increased neuronal expression of Zif268 in the superficial lamina of the dorsal horn and that spinal Zif268 antisense

treatment results in deficits of LTP in dorsal horn neurones following peripheral injury (Rygh et al., 2006).

1.9.2 LTP and nociceptive circuits

LTP displayed by spinal neurones is mediated by the release of glutamate and SP from central primary afferent terminals, which causes the opening of NMDAR channels and T-type VDCCs as well as Ca^{2+} release from intracellular stores. This activates Ca^{2+} dependent signal transduction pathways that involve protein kinases and transcription factors. Synaptic strength is increased e.g. by phosphorylation of synaptic proteins including AMPARs; altered trafficking of synaptic proteins and de novo protein synthesis. These are changes that act to facilitate neurotransmitter release and/or conduction (Bliss and Collingridge, 1993; Sandkuhler, 2007). These changes result in an increased responsiveness of spinal neurones and are believed to be pivotal in contributing to clinical hyperalgesia and allodynia (D'Mello and Dickenson, 2008).

Bliss and Lomo were the first to describe the most frequently used form of conditioning stimulation to induce hippocampal LTP, that consists of high frequency electrical stimulation (HFS) of an input pathway (Bliss and Lomo, 1973). Relevant to pain, Liu and Sandkuhler were the first to report that LTP of C-fibre evoked field potentials in the superficial dorsal horn in vivo may last for more than 8 hr (Liu and Sandkuhler, 1995). This was achieved by stimulating the sciatic nerve at a frequency of 100 Hz, given in 4 trains lasting 1 s at 10 s intervals i.e. high frequency burst-like activity. Furthermore, the spinal LTP evoked was blocked using the NMDA antagonist D-(-)-4-(3-phosphonopropyl)piperazine-2-carboxylic acid (DCPP) confirming the necessity for NMDAR mediated Ca^{2+} influx. It has also been found that repetitive HFS of primary afferents not only results in LTP of superficial dorsal horn neurones that is C-fibre mediated, it also induces LTP mediated by Aδ-fibres (Randic et al., 1993).

As well as inducing LTP with high frequency electrical stimuli, LTP mediated by nociceptive C-fibres can also be induced by noxious stimuli that are directly responsible for producing clinical allodynia and hyperalgesia including skin burns,

contusions, inflammation and acute nerve injury (Sandkuhler and Liu, 1998). This particular study by Sandkuhler and Liu as well as other LTP experiments carried out by the same group were performed on spinalised rats whereby, after an injection of the anaesthetic lidocaine into the third segment of the cervical region of the spinal cord, the cord is then cut and the rats consequently mechanically ventilated. However, LTP can also be induced in deep WDR neurones in intact rats (Svendsen et al., 1997; Rygh et al., 1999), although LTP of C-fibre evoked responses is larger and easier to obtain in spinalised rats (Rygh et al., 2002). The fact that LTP can also be recorded from WDR neurones means that the effects of LTP-induction are not being filtered out at other levels of the neuraxis. In addition, the augmented LTP observed in spinalised rats confirms that LTP of WDR neurones is mainly localised to the spinal neurones, yet can be modulated by descending pathways under physiological conditions.

It is important to note that spinal LTP is different from the wind up discussed in section 1.4.4. In the rat, LTP is induced by high frequency (100 Hz) electrical stimulation of the sciatic nerve, resulting in enhanced responses of dorsal horn WDR neurons to single Aδ- or C-fibre stimuli for up to 6 hr (Svendsen et al., 1997). In contrast, wind up is induced by lower stimulation frequencies (0.3 – 2 Hz) and the enhanced responsiveness may maximally last for a few minutes after the end of a stimulation train. Whilst wind up has been used to explore putative mechanisms underlying central sensitization, there is still some debate as to whether the study of LTP is useful for examining mechanisms of centeral sensitization due to the substantially high frequencies used to induce the state (Eide, 2000).

1.9.3 LTP and protein synthesis

The persistence of LTP and thus learning and memory has been shown to require new protein synthesis. Furthermore, it is believed that these newly synthesised proteins are targeted by two main mechanisms that specifically target these proteins to activated synapses. One mechanism, involves synthesis of proteins within the cell body, which are then captured by active 'tagged' synapses therefore facilitating responses to further stimuli at those synapses. The second mechanism involves the synthesis of proteins locally in neuronal dendrites at the

site of synaptic activation (Martin and Kosik, 2002; Bailey et al., 2004; Kelleher et al., 2004).

In 1984, Krug et al. were the first to report a requirement for newly synthesised proteins in long lasting hippocampal LTP (Krug et al., 1984). In their studies, freely moving rats were chronically implanted with a stimulation electrode into the medial entorhinal cortex and a recording electrode into the dentate gyrus of the hippocampus in order to induce and record LTP with a tetanising stimulus. When they administered an intraventricular dose of the global translation inhibitor anisomycin (which inhibits ribosomal machinery and thus the decoding of messenger ribonucleic acid (mRNA) in the cytoplasm of a cell), there was no effect on the induction of LTP, yet long lasting LTP (3 hr - 7 d) was significantly attenuated.

To date, there have been many detailed studies that have identified a variety of signalling pathways and phosphorylation events that link synaptic activity to changes in the activity of various regulatory transcriptional proteins i.e. proteins that are involved in converting nuclear deoxyribonucleic acid (DNA) to mRNA during protein synthesis. One such transcriptional factor is cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). This transcription factor is induced in many cell types in response to extracellular signals such as neurotransmitters. These signals cause translocation of CREB to a short sequence of DNA within the promoter of specific genes within the nucleus i.e. the CRE. In the nervous system, targets for CREB include genes for BDNF and the NMDAR-coupled signalling molecule neuronal nitric oxide synthase (nNOS) as well as others (West et al., 2002).

Experiments on the Californian sea slug (*Aplysia californica*) have perhaps been the most revealing in terms of mechanisms that mediate neuronal hyperexcitability such as that seen with LTP. These animals are ideal for physiological research because they have a relatively simple nervous system consisting of a few thousand easily identifiable neurones. Key studies in *Aplysia* have demonstrated synaptic tagging in a culture system where a single bifurcated *Aplysia* sensory neurone is in contact with two spatially separated motor neurons (Casadio et al., 1999). In these

studies, a single puff of 5-HT was delivered to one contact and five puffs were delivered to the other contact. A single puff was found to produce only transient facilitation whereas if five puffs were first applied to the other contact, then the facilitation produced by the single puff was longer lasting. This increase in synaptic potency persisted for more than 24 hr and was dependent upon transcription as shown by inhibition with the transcriptional inhibitor actinomycin D. It was confirmed that transcription was mediated by CREB due to the fact that injection of anti-CREB antibodies into the neurone cell body blocked further facilitation at the tagged synapse. Of particular interest, was the identification of two components of the tagging system. Initiation of facilitation as well as facilitation produced within the first 24 hr from a single puff of 5-HT was dependent upon protein kinase A (PKA), which is upstream of CREB, yet this pathway was not sufficient for the self-maintained stabilisation of the plastic changes that persisted beyond 72 hr. Instead, this was found to be dependent on specific protein translation pathways that were sensitive to the drug rapamycin, the inhibitor of the mammalian target of rapamycin (mTOR). In the past few years, there has been much interest in the roles of these pathways and particularly, their roles in neuronal plasticity.

1.10 Rapamycin-sensitive pathways

The serine-threonine protein kinase mTOR was identified and cloned shortly after the discovery of two yeast genes, TOR1 and TOR2, in the budding yeast *Saccharomyces cerevisiae* during a screen for the resistance to the immunosuppressant drug rapamycin (Hay and Sonenberg, 2004). mTOR can be autophosphorylated via its intrinsic serine/threonine kinase activity and it regulates protein synthesis through the phosphorylation and inactivation of the repressor of mRNA translation, eukaryotic initiation factor 4E (eIF4E)-binding protein (4EBP) and through the phosphorylation and activation of the 70 kDa ribosomal protein S6 kinase (p70S6K) (Brown and Schreiber, 1996). Phosphorylation of mTOR as well as p70S6K and 4EBP phosphorylation is commonly used as an activity read-out of these pathways, which can be inhibited by the drug rapamycin.

Rapamycin is produced by the soil bacterium *Streptomyces hygroscopicus* and was originally purified as an antifungal agent after its discovery from the soil of Easter Island (Vezina et al., 1975). However, it was deemed unsuitable due to its undesirable immunosuppressive side effects. After investigating further into these side effects, rapamycin is now often the drug of choice to prevent rejection after kidney transplantation due to its inhibitory effects on T-cell growth and IL2-induced proliferation (Cohen, 2002). Furthermore, rapamycin was the first drug to be approved for clinical use that selectively inhibits one protein kinase (Liu et al., 1991). Rapamycin forms an inhibitory complex with its intracellular receptor, the FK506-binding protein (FKBP12), which binds a region in the C terminus of mTOR proteins, thereby inhibiting mTOR activity (Hay and Sonenberg, 2004).

mTOR regulates several intracellular processes in response to various extracellular signals, nutrient availability, energy status of the cell and stress. Relevant to nociceptors is the fact that rapamycin-sensitive pathways are known to regulate survival, differentiation and development of neurones. It is therefore of no surprise that mTOR activity is modified in a wide range of pathological states including neurodegenerative disorders such as Alzheimer's disease (Jaworski and Sheng, 2006; Swiech et al., 2008).

The canonical pathway for mTOR activation begins with the activation of receptor kinases by mitogens such as lipopolysaccharide, trophic factors such as BDNF or hormones such as insulin. This leads to the activation of phosphoinositide-3 kinase (PI3K) via the Ca^{2+}-dependent recruitment of the protein Ras (as well as other adaptor proteins) and increased production of phosphatidylinositol 3,4,5-triphosphate (PIP3) via phosphorylation of phosphatidylinositol 4,5-biphosphate (PIP2). PIP3 can be converted back to PIP2 via the action of phosphatase and tensin homolog deleted on chromosome 10 (PTEN). The increase in PIP3 results in the recruitment of 3-phosphoinositide-dependent protein kinsae 1 (PDK1) and the serine-threonine kinase Akt to the membrane and the subsequent phosphorylation of Akt by PDK1. Akt can then phosphorylate tuberin (TSC2) which, together with hamartin (TSC1) forms the tuberous sclerosis complex TSC1/2. This acts as a guanine triphosphate (GTP)-ase activating protein (GAP) for Rheb (Ras homolog enriched in brain protein). Inactivation of TSC2, caused by Akt-mediated phosphorylation, as well as activation of the guanine exchange factor for Rheb-translationally controlled tumour protein (TCTP), results in increased Rheb-GTP levels in the cell and this has a stimulatory effect on mTOR activity (Swiech et al., 2008). Together with its binding partner raptor, mTOR controls translation via the phosphorylation of both 4EBP and p70S6K. This leads to the activation of a number of targets involved in mRNA translation (Takei et al., 2004) (see figure 1.4). As well as the canonical pathway, there are also other upstream regulators of mTOR at the level of other signalling systems, receptors and neurotransmitters that have been shown to engage rapamycin-sensitive pathways. Many of these components have received much attention in the past few years and are summarised in tables 1.3 and 1.4.

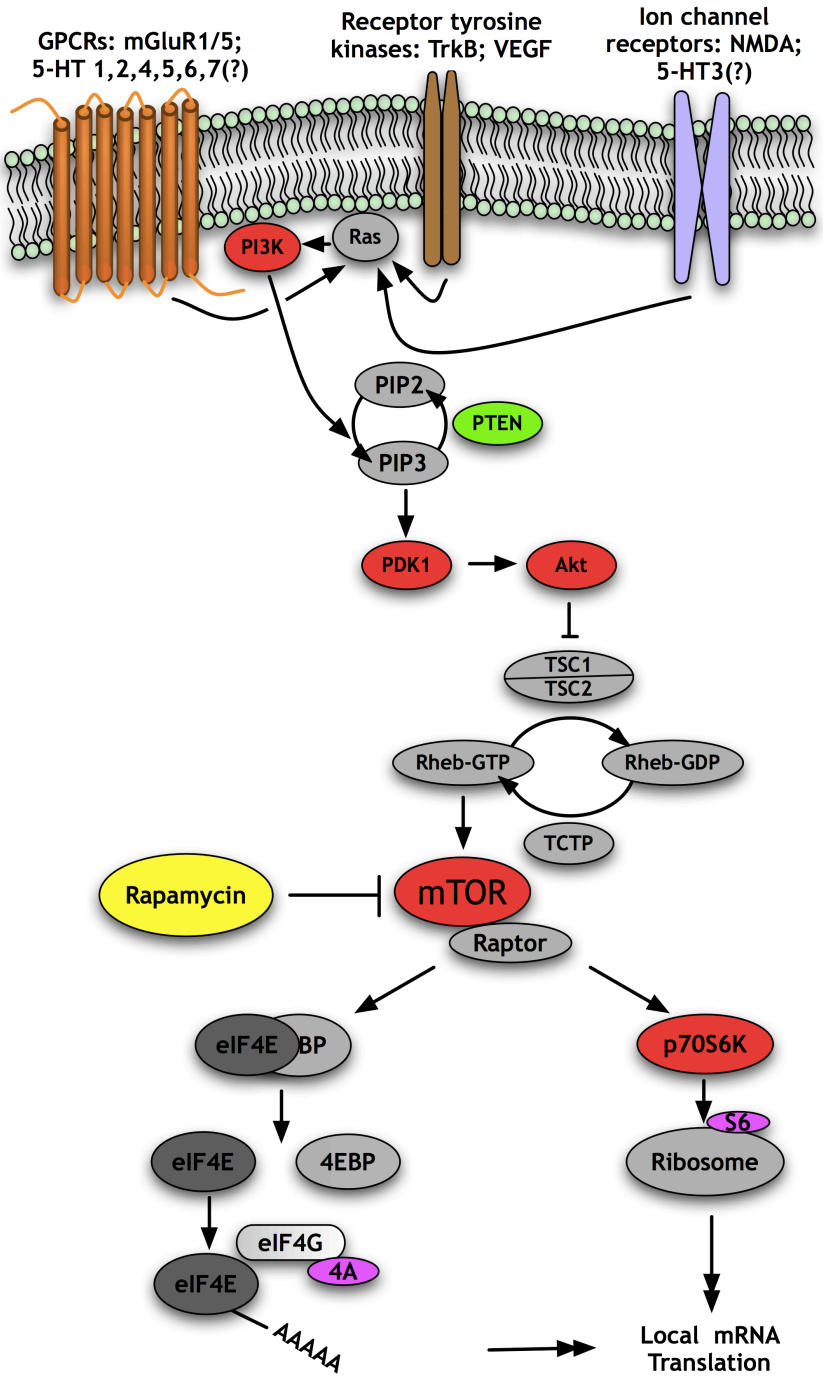


Figure 1.4 Schematic diagram of mTOR activity control. Stimulation of several receptors at the plasma membrane by mitogens, trophic factors and neurotransmitters leads to mTOR activation via Ras- and PI3K-dependent pathways. The activity of mTOR can be specifically inhibited by the drug rapamycin. Arrows indicate activation and bars indicate inhibition (adapted from Takei et al., 2004; Swiech et al., 2008).

Ligand/Receptor	Evidence
Glutamate/NMDARs	NMDARs modulate mTOR/p70S6K translation-related proteins (Gong et al., 2006; Gonzalez-Mejia et al., 2006; Yoon et al., 2008). Cationic amino acid transporters mediate NMDAR activation-dependent changes via the mTOR pathway (Huang et al., 2007). NMDARs engage mTOR pathways and are required for LTP (Cammalleri et al., 2003).
Glutamate/mGluRs	Protein translation via mTOR involves mGluR1/5 receptors and contributes to behavioural hypersensitivity (Price et al., 2007). Long-term depression involves mGluR1/5-mTOR signaling (Hou and Klann, 2004; Banko et al., 2006; Page et al., 2006).
5-HT/5-HTRs	5-HT-induced facilitation is mediated by mTOR signalling (Casadio et al., 1999; Khan et al., 2001; Carroll et al., 2004; Hu et al., 2006; Hu et al., 2007; Weragoda and Walters, 2007).
BDNF/TrkB	BDNF-induced neuronal protein translation is mediated by mTOR (Takei et al., 2001; Schratt et al., 2004; Takei et al., 2004).
Neurotrophin 3 (NT3)/TrkC, TrkB, low affinity nerve growth factor receptor (LNGFR)	NT3-mediated LTP requires mTOR signalling (Je et al., 2005).
Vascular endothelial growth factor (VEGF)/VEGF receptor	VEGF signalling activates mTOR (Kim et al., 2008).

Table 1.3 Recent progress on neurotransmitters and receptors that engage rapamycin-sensitive pathways.

Signalling molecule	Evidence
PI3K	PI3K activation engages rapamycin-sensitive pathways that are implicated in neuronal development and plasticity (Takei et al., 2001; Schratt et al., 2004; Horwood et al., 2006; Hu et al., 2006; Chenal and Pellerin, 2007; Hu et al., 2007; Kelly et al., 2007; Tsokas et al., 2007).
PKC	PKC activation engages rapamycin-sensitive pathways that are implicated in neuronal plasticity (Khan et al., 2001; Kelly et al., 2007).
Extracellular signal regulated kinase (ERK)	ERK activation engages rapamycin-sensitive pathways that are implicated in neuronal plasticity (Gelinas et al., 2007; Kelly et al., 2007; Tsokas et al., 2007).
Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII)	CaMKII activation engages rapamycin-sensitive pathways that are implicated in neuronal development and plasticity (Schratt et al., 2004; Kelly et al., 2007).

Table 1.4 Recent progress on neuronal intracellular signalling molecules that engage rapamycin-sensitive pathways.

1.10.1 Multi-signalling convergence upon mTOR

The information in tables 1.3 and 1.4 gives the impression that there are group of specific neurotransmitters, receptors and intracellular signalling pathways that engage rapamycin-sensitive pathways. However, given what is already known about the complexity and diversity of intracellular signalling pathways, this is not the case. For example, whereas ERK-dependent pathways and PI3K-dependent pathways will both activate mTOR, at the receptor level, they can both be indirectly regulated by the same transmitters/receptors and can converge before they reach mTOR (Tsokas et al., 2007; Swiech et al., 2008). Essentially, there are a number of cellular processes that act to enhance or inhibit neuronal excitability that converge on mTOR. The information in tables 1.3 and 1.4 merely represents recent progress on some of the most characterised intracellular signalling molecules relevant to neuronal excitability.

Relevant to pain, many of the neurotransmitters, receptors and signalling molecules that engage mTOR pathways, have been shown to have key roles in nociception and persistent pain-like states. CaMKII and ERK have been shown to play important roles in mediating behavioural hypersensitivity to formalin-induced inflammation or nerve injury (Zhuang et al., 2005; Choi et al., 2006). BDNF has been shown to be important in mediating formalin-induced inflammation (Kerr et al., 1999) as well as nerve injury-induced behavioural hypersensitivity (Yajima et al., 2005). Likewise, there are numerous reports on the importance of the action of glutamate on NMDARs and mGluRs in mediating the neuronal changes and behavioural hypersensitivity associated with persistent pain-like states (Haley et al., 1990; Chaplan et al., 1997; Dogrul et al., 2000; Suzuki et al., 2001; Varty et al., 2005)

Importantly, rapamycin-sensitive pathways exert significant effects on neuronal excitability that can be modulated by neurotransmitters, receptors and signalling molecules that are known to play crucial roles in persistent pain-like states. However, there have been few studies that have specifically investigated the importance of these pathways in nociception and persistent pain-like sates.

1.11 Rapamycin-sensitive pathways and neuronal excitability

Key studies on *Aplysia* and hippocampal-induced LTP have revealed important aspects of rapamycin-sensitive pathways and neuronal excitability. Neurones from *Aplysia* have been used to test the hypothesis that axonal injury (crush) or the focal depolarisation that accompanies axonal injury (using a high extracellular K^+ treatment) can trigger a local decrease in action potential threshold and result in long-term hyperexcitability (LTH) (Weragoda et al., 2004). In this study, nociceptive tail sensory and motor nerve axons exhibited LTH lasting at least 24 hr after crushing or an elevated extracellular K^+ treatment. Furthermore, exposure of the nerve axons to the global protein translation inhibitor anisomycin and rapamycin resulted in prevention of the LTH induced by both treatments. This dependence on protein synthesis was found to be state specific because thresholds of axons in uninjured or untreated nerves were unaffected by the inhibitors

The crush-induced LTH was later found to be mediated by 5-HT (Weragoda and Walters, 2007) because application of the 5-HT receptor antagonist methiothepin to nerve segments inhibited this crush-induced LTH. In addition, the 5-HT-induced LTH was also inhibited by rapamycin. These studies are of particular interest because of the involvement of 5-HT as a nociceptive signal within the peripheral and central nervous systems. Further substantiating the link between 5-HT and rapamycin-sensitive pathways is the finding that 5-HT application decreases the phosphorylation of the elongation factor downstream of mTOR, eEF2 thus enabling subsequent protein translation (Carroll et al., 2004).

The importance of rapamycin-sensitive pathways in neuronal plasticity has been well demonstrated by in vitro studies involving hippocampal LTP. Disruption of mTOR signalling by rapamycin has been shown to reduce LLTP, whilst not affecting the early phase. In addition, these effects were mediated at the postsynapse because immunoreactivity for the presynaptic marker synapsin-I opposes and slightly overlaps immunoreactivity for rapamycin-sensitive pathways. Immunoreactivity for rapamycin-sensitive pathways showed that these pathways were present in both cell body (stratum pyramidale) and dendritic (stratum

radiatum) structures of the CA1 hippocampal region (Tang et al., 2002). Following on from these studies, Cammalleri et al. determined exactly when during LLTP that rapamycin-sensitive pathways are important. In their studies, they showed that rapamycin prevents LLTP only when applied during induction. Specifically, when rapamycin was applied transiently to hippocampal slices for 40 min during LLTP induction, there was a significant decrease in LLTP. However, a transient rapamycin application 5 min after the induction of LLTP or 2 hr after the completion of LLTP had no effect. In addition, it was found that p70S6K phosphorylation was dependent on NMDARs and PI3K. Also of interest was the discovery that immunoreactivity for the phosphorylated form of p70S6K was very low in the dendrites of CA1 hippocampal neurones until there was a delivery of the LLTP-inducing stimulus, after which immunoreactivity for phosphorylated p70S6K increased in the dendrites but not the cell bodies of the neurones (Cammalleri et al., 2003).

LTP has also been shown to increase the expression of another elongation factor downstream of mTOR, eEF1A. This is a process that is blocked by rapamycin (Tsokas et al., 2005). As well as the link between mTOR and PI3K, LTP has also been used to demonstrate the interaction between mTOR and ERK (Tsokas et al., 2007). In this study, LTP-inducing stimuli resulted in an increase in mTOR signalling that was blocked by inhibiting ERK signalling. The concept of ERK playing an important part in regulating mTOR is not in line with the canonical mechanism described earlier (see figure 1.4). However, it was found that ERK inhibition also resulted in a decrease in phosphorylation of proteins involved in the canonical PI3K-mTOR pathway. These proteins were PDK1 and Akt. This suggests that mTOR-mediated protein synthesis in LTP requires mutually dependent and concomitant activity of ERK and PI3K pathways.

In neurodegenerative disease, rapamycin-sensitive pathways have been studied in a transgenic model of Alzheimer's disease (Damjanac et al., 2008). In these studies, mice that had a mutation of amyloid precursor protein (APP) and presenilin- two proteins heavily implicated in Alzheimer's disease, demonstrated a reduction in activity of mTOR signalling at the level of p70S6K. These differences were specific for phosphorylated p70S6K and activation of ERK1/2 and 4EBP1 were not modified.

In addition, mTOR levels were not modified, yet there was a robust accumulation of phosphorylated Akt, thus demonstrating a dissociation between Akt and mTOR signalling markers such as p70S6K and confirming the complexity of subcellular signalling involving mTOR.

1.11.1 Rapamycin-sensitive pathways and persistent pain

Relevant to pain, protein synthesis has been found to be an important component of the behavioural hypersensitivity associated with formalin-induced inflammation (Kim et al., 1998). In this study, the protein transcription inhibitor actinomycin and the protein translation inhibitor anisomycin were found to attenuate formalin-induced hypersensitivity when administered prior to the application of formalin to the hind paw of mice. Rapamycin-sensitive pathways in particular have also been implicated in formalin-induced hypersensitivity in mice lacking the fragile X mental retardation gene (FMR1), another protein that influences protein translation in neurones (Price et al., 2007). In these studies, it was found that FMR1 was important for pain processing, since knock out (KO) mice displayed reduced hypersensitivity to formalin-induced inflammation compared to their wild type (WT) littermates. Furthermore, rapamycin was ineffective in reducing formalin-induced behavioural hypersensitivity as well as thermal hyperalgesia as a result of the spinal administration of the mGluR1/5 agonist (RS)-3,5-dihydroxyphenylglycine (DHPG) in FMR1 KO mice compared to their WT littermates.

More recently, components of rapamycin-sensitive pathways have been identified in myelinated nociceptive sensory fibres (fast conducting Aδ-fibres) at the level of the hind paw in rat cutaneous tissue. In addition, electromyograhy (EMG) showed that rapamycin inhibited the sensitivity of fast conducting Aδ-fibres since the withdrawal threshold to noxious heat in anaesthetised rats was significantly increased for the fast conducting Aδ-fibres rather than the slow conducting, but nociceptive-specific C-fibres. Furthermore, behavioural studies showed that local (hind paw) treatment with rapamycin, attenuated persistent capsaicin-induced hypersensitivity akin to clinical secondary or referred hyperalgesia (Jimenez-Diaz et al., 2008). Taken together, these studies implicate mTOR as a key player in pain processing.

1.12 Thesis aims

Presently, there are a wide range of drugs that are currently used to treat acute and persistent pain syndromes such as NSAIDs, opioids and antidepressants. Yet many of these drugs are only often partially effective and can often be accompanied by unpleasant side effects or subject to abuse. In the past decade, there has been an intensive drive to determine the molecular and genetic signatures of persistent pain states with the hope of developing improved therapies.

Although the pain studies investigating mTOR have identified a potential target important in pain processing, there is still much to be discovered. The aims of this thesis are as follows:

1. To use in vivo electrophysiology to characterise neuronal responses to peripheral stimuli in naive rats and also rats where a persistent pain-like state (based on clinical neuropathy and inflammation) has been induced. The aim here is also to understand how spinal rapaymycin-sensitive pathways influence these responses.

2. To use behavioural studies to characterise behavioural hypersensitivity in rat persistent pain models and to understand how rapamycin-sensitive pathways influence this behaviour.

3. To use immunohistochemistry to determine the distribution of rapamycin-sensitive pathways at the spinal level and to determine how these pathways are affected in rat models of persistent pain.

4. To use pharmacological agents to probe descending facilitatory pathways from higher brain centres in persistent pain states and determine what effect, if any these pathways have on rapamycin-sensitive pathways at the spinal level.

2 Methods

2.1 In vivo electrophysiology recordings from spinal cord neurones

2.2 Animals

For all studies, male Sprague Dawley rats (250 – 280 g) were used. These were supplied by the Biological Services Unit (BSU, University College London, UK). All procedures described were approved by the Home Office (UK) and were in agreement with the IASP guidelines (Zimmermann, 1983).

2.2.1 Set up

In vivo electrophysiology studies were carried out according to a well established protocol (Urch and Dickenson, 2003). Rats were initially anaesthetised in an induction box with 4 % isofluorane in a mixture of nitrous oxide (66 % v/v) and oxygen (33 % v/v). Once the rats had lost consciousness and were completely areflexic, the trachea was exposed and isolated and a tracheal cannula was inserted into the trachea and fastened with 3-0 silk threads. This was used to maintain anaesthesia throughout the recording period. At this stage, the isofluorane was reduced to 2.5 % v/v (areflexia was maintained). Rats were then secured in a stereotaxic frame and a rectal probe attached to a heating blanket was used to maintain a core temperature of 37 °C.

An incision was made through the skin along the length of vertebrae and the skin was then separated from the underlying muscle. Muscle, connective tissue and vertebrae were specifically removed from lumbar vertebral segments L1 – L3 of the spinal cord. Muscle and connective tissue from surrounding areas were kept intact and this created a well in the exposed spinal cord area into which, drug solutions could be added. Clamps were used to stabilise and straighten the cord. The dura mater was also removed to aid drug penetration. When the set up was complete, the isofluorane was reduced to 1.8 % v/v, a level sufficient for anaesthesia, whilst

maintaining areflexia. All drugs were applied directly onto the exposed spinal cord in a volume of 50 μl.

2.2.2 Recording system

Recordings were obtained with an AC recording system (NeuroLog system, Digitimer, UK). An electrode (parylene insulated tungsten microelectrode, 125 μm diameter, 2 MΩ, A-M systems Inc., USA) inserted into a head stage attached to a 3-axis manipulator was manually lowered into the exposed cord (L4 - L5) to a depth of 500 – 1000 μM. This is an area occupied by WDR neurones, which were initially characterised and located by tapping the receptive field of the ipsilateral hind paw of the rat (varying from the foot pad of the rat to the toes). The recording system was grounded via a lead connected to the stereotaxic frame and a second lead connected to the rat. The signal from the rat was subtracted from the input form the electrode using the Neurolog differential recording mode to reduce interference. Neuronal activity was amplified, filtered, and then displayed on an oscilloscope as well as being made audible via a speaker system. Quantification of neuronal activity was achieved with a 1401 interface and Spike 4 software (Cambridge Electronic Design or CED, UK) (see figures 2.1 and 2.2). Once a single neurone had been isolated, based on a single amplitude series of action potentials, a common shape and a good signal to noise ratio, a number of stimuli were then applied to the receptive field.

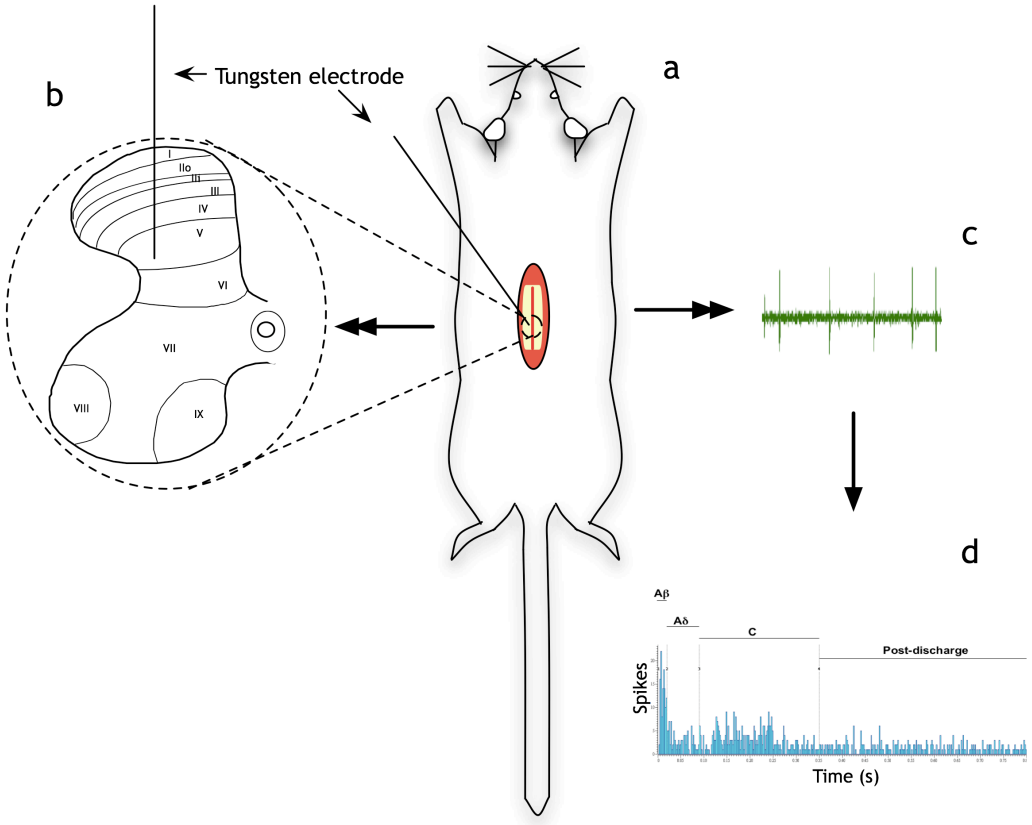


Figure 2.1 Overview of in vivo electrophysiology setup. (a) Laminectomy and destination of electrode in the dorsal horn. (b) Close up of electrode in the dorsal horn (recording from neurones in lamina V - VI). (c) Digital recording of neuronal activity or spikes. (d) Example stimulus response profile comprising the different components of the inputs that converge on the recorded neurone produced by electrical stimulation (0.5 Hz) at three times the C-fibre threshold. A stimulus response profile was also produced by a range of 'natural' stimuli including mechanical (brush, pinprick and von Frey filaments) and thermal (water jet) stimuli applied for 10 s each.

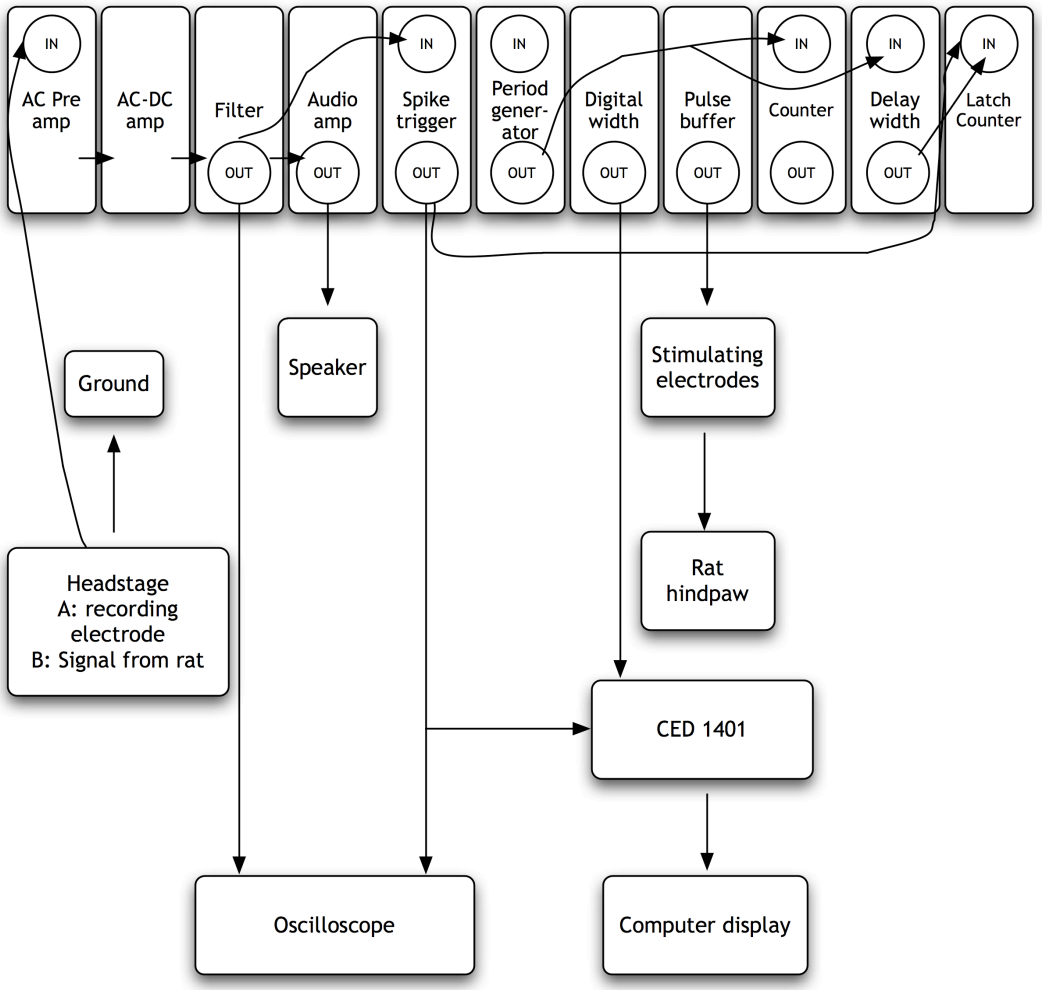


Figure 2.2 Neurolog data capture system. The recording electrode is inserted into the spinal cord (A) and a lead connected to the rat (B). A second lead is used to ground the streotaxic frame. The signal is then fed into the NeuroLog data capture system, differentiated as A-B, amplified and fed into an audio speaker and oscilloscope. Action potentials above set amplitudes are discriminated and fed into the computer system via the CED 1401 interface. Electrical stimuli can be administered via the stimulating electrodes in the neuronal receptive field. The frequency of stimulation, duration, amplitude of the current and number of pulses are set with the period generator, digital width, pulse buffer and counter. The number of action potentials evoked for a set time frame (90 - 800 ms), are displayed on the latch counter and on the computer display. Natural stimuli are applied with increasing intensity to the receptive field and the resulting action potentials are displayed visually on a rate histogram and as numerical action potentials per 10 s (adapted from Urch and Dickenson, 2003).

2.2.3 Characterising and quantifying stimulus evoked responses

Punctate mechanical stimuli were delivered by applying graded von Frey filaments or a pin, to the most sensitive part of the receptive field for 10 s (with the pinprick stimuli, care was taken not to break the skin). This was also the case for dynamic stimuli, where an artist's brush was used and thermal stimuli, where increasing heat was applied using a jet of water from a 60 ml syringe attached to a needle (see figure 2.3 for typical neuronal responses). Previous studies have shown that in naive rats, the 50 % behavioural mechanical withdrawal threshold varies from around 11 g to 19 g (Chaplan et al., 1994; Baik et al., 2003). For thermally evoked responses, the nociceptive threshold is approximately a 45 °C applied stimulus (Hargreaves et al., 1988). Therefore for the purpose of these studies, all mechanical stimuli $\geq$ 15 g and thermal stimuli $\geq$ 45 °C are classified as noxious stimuli and all stimuli below these values are classified as innocuous stimuli.

Electrical stimuli were delivered by inserting two stimulating electrodes intradermally into the most sensitive part of the receptive field. Firstly, Aβ- and C-fibre thresholds were determined depending on their latencies to respond to stimuli (Aβ-fibres = <20 ms post-stimulus; C-fibres = 90 - 300 ms post-stimulus). The stimulator was then set to three times C-fibre threshold and a train of 16 stimuli (0.5 Hz, 2 ms pulse width) was delivered to the receptive field to determine the number of action potentials attributable to Aβ-fibres (0 – 20 ms); Aδ-fibres (20 – 90 ms); C-fibres (90 – 300 ms) and post-discharge (300 – 800 ms) which is attributable to the wind up elicited by repeated stimuli of nociceptive C-fibres. The input (non-potentiated response) and the wind up (potentiated response) were calculated as follows: C-fibre Input = action potentials (90 - 800 ms) evoked by the first pulse at three times C-fibre threshold multiplied by the total number of pulses (16). This then represents the theoretical baseline in the absence of wind up. Wind up = total action potentials (90 – 800 ms) after the 16-train stimulus at three times C-fibre threshold minus the input. This represents the excess activity above the theoretical baseline due to wind up. Only stable cells where 3 consecutive stimulus evoked responses were within 10 % of the previous result for the same test were selected for further pharmacological study.

At the end of all experiments, rats were overdosed on 5 % isofluorane and a cervical dislocation of the neck was performed to ensure death

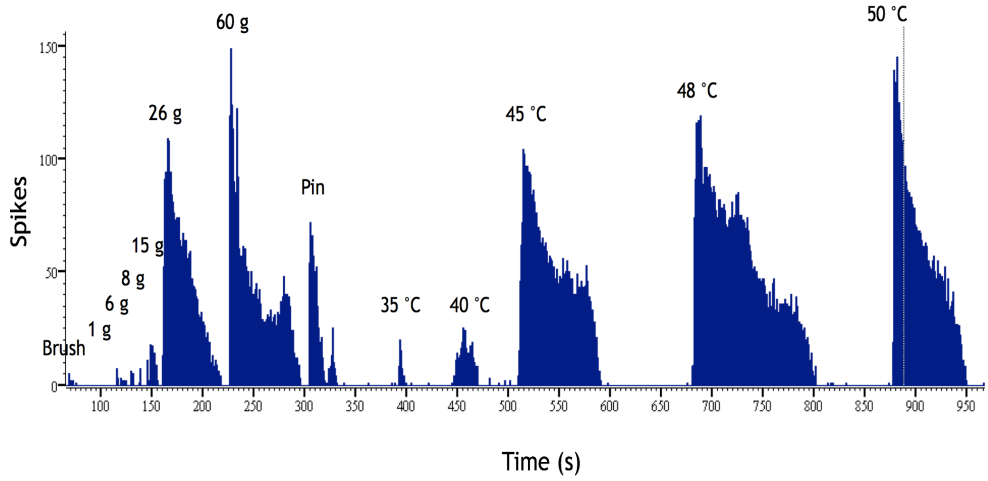


Figure 2.3 Example neuronal responses to natural stimuli. Stimulus histogram showing neuronal responses to mechanical stimuli (brush, graded von Frey filaments and pin prick) and graded thermal stimuli (water jets) applied for 10 s. The ordinate shows the total number of spikes within a 1 s bin.

2.3 Formalin-induced inflammation model

The formalin test was carried out based on a well established protocol (Dubuisson and Dennis, 1977).

2.3.1 In vivo electrophysiology recordings from spinal cord neurones

See 2.1. To monitor spontaneous neuronal activity as a result of formalin-induced inflammation, a 5 % v/v formalin solution made from 40 % v/v formaldehyde solution (BDH Chemicals Ltd, UK) was intradermally injected into the hind paw (50 μl) ipsilateral to the WDR neurone, which had already been characterised using a 0.5 ml insulin syringe (BD Micro-Fine™). A WDR neurone was selected on one side of the cord for treatment with spinally administered (intrathecal or i.t.) vehicle prior to formalin injection into the corresponding hind paw. Only after a biphasic control response was achieved was a neurone then selected on the opposite side for treatment with the drug prior to formalin injection into the corresponding hind paw. Neuronal activity was separated into bins of 10 min.

2.3.2 Assessment of the effectiveness of drugs on pain-like behaviour

Before each behavioural study, each rat was allowed to acclimatise for 30 min in individual open top clear Plexiglass chambers (length, width, height = 25 x 25 x 25 cm). In order to determine the effect of drugs at the spinal level on pain-like behaviour, rats were first lightly anaesthetised on 2 % v/v isofluorane in a mixture of nitrous oxide (50 % v/v) and oxygen (50 % v/v) after which they were disinfected and lightly shaved across their backs. A 0.5 ml insulin syringe (BD Micro-Fine™) was used to inject a 20 μl i.t. dose of drug solution through the skin, into the L5 – L6 vertebral interspace, after which the rats were allowed to recover prior to formalin injection into the hind paw. A 20 μl volume has been shown to produce uniform coverage of the spinal cord which is restricted to the sacral and cauda equina levels and extends up to thoracic T13 – lumbar L1 (Xu et al., 2006). After the rats had recovered, they were restrained and 5% v/v formalin solution was then

administered to the left hind paw. The rats were then placed back into their chambers and observed for 1 hr. The following behaviours were measured: 1) licking and biting and 2) lifting and flinching (Sufka et al., 1998). Behavioural data were separated into bins of 5 min. The drug regimen was blinded until the analysis was complete. After all behavioural studies, rats were overdosed on a rising concentration of CO_2, after which, death was ensured by cervical dislocation of the neck.

2.4 Spinal nerve ligation model

2.4.1 Surgery

The spinal nerve ligation (SNL) model was produced by following a well established protocol (Kim and Chung, 1992). Rats (140 - 160 g) were initially anaesthetised in an induction box with 4 % v/v isofluorane in a mixture of nitrous oxide (50 % v/v) and oxygen (50 % v/v). After this, the isofluorane was reduced to 2.5 % (areflexia was maintained). The rats were then cleaned with disinfectant and shaved across their back. A heating blanket was placed underneath the rats (which were placed in a prone position) that maintained a core temperature of approx 37 ºC.

An incision was made through the skin along the length of vertebrae and the skin was then separated from the underlying muscle. The left paraspinal muscles were separated from the spinous processes at the L4 - S2 levels. The L6 transverse process was then partially removed with rongeurs to allow visual identification of the L4, L5 and L6 spinal nerves. The left L5 and L6 spinal nerves were then isolated and tightly ligated with 6-0 silk threads. The same procedure was performed in sham animals, except for ligation of the spinal nerves. A complete homeostasis was confirmed before the wound was then sutured (See figure 2.4).

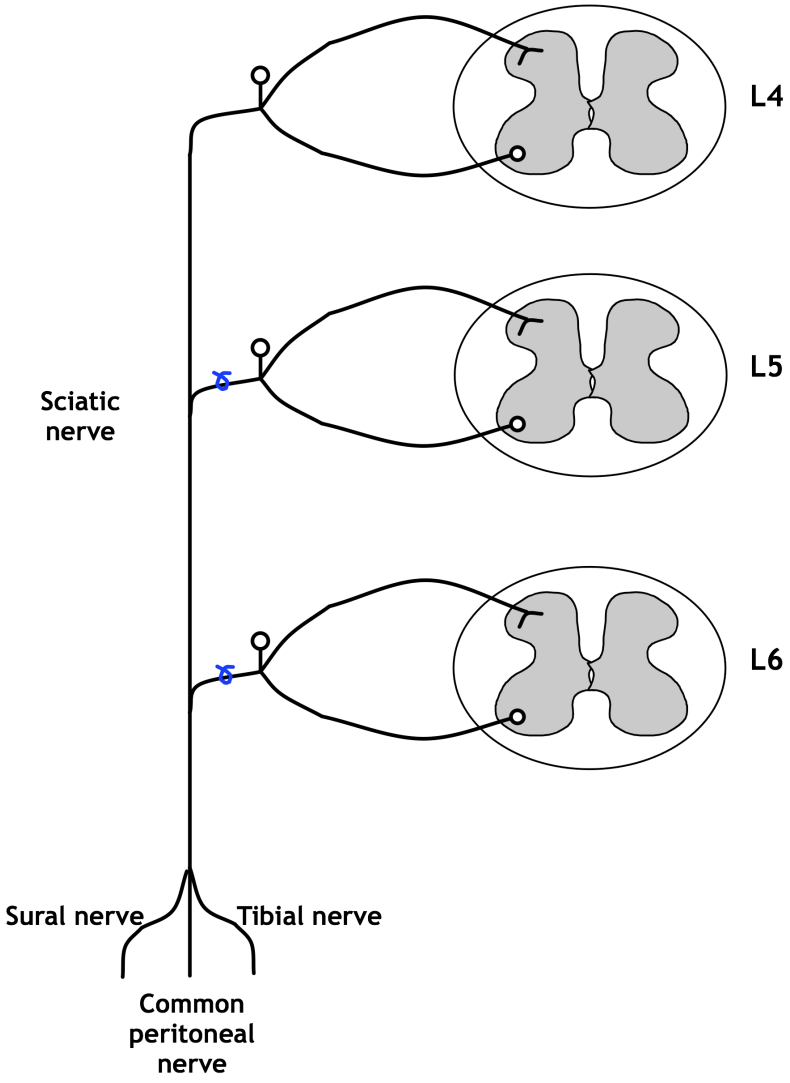


Figure 2.4 Schematic of spinal nerve ligation procedure. Spinal nerve ligation is produced by the tight ligation (blue) of the spinal nerves distal to the dorsal root ganglion at L5 and L6.

2.4.2 Assessment of the progression of pain-like behaviour

Rats were tested on days 2, 7, 9 and 14 to assess the progression of pain-like behaviour. Prior to testing, animals were allowed to acclimatise to the room in their cages for at least 15 min. They were then transferred to a row of adjoining plastic testing chambers (length, width, height = 10 cm x 12.5 x 25 cm) on a wire-mesh platform where they were allowed to acclimatise for a further 15 min. The rats were then tested with innocuous mechanical stimuli comprising 1, 6 and 8 g von Frey filaments (Touch-test™, North Coast Medical Inc., USA) and a cooling stimulus comprising acetone in a modified syringe attached to plastic tubing (see figure 2.5). The maximal force of each von Frey filament was applied to the ipsilateral and contralateral hind paw a total of 10 times (to the most responsive area of the receptive field determined with the 6 g von Frey filament). Acetone was applied to the hind paw a total of 5 times by expelling 0.5 ml drop through a 1 ml syringe attached to a piece of tubing onto the hind paw, taking care not to touch the paw with the tubing. Stimuli were applied for a total of 2 s and each set was separated by at least 3 min. The testing sequence for each stimulation set was as follows:

1. 1 x acetone to each hind paw.
2. 10 x von Frey 1 g to each hind paw.
3. 1 x acetone to each hind paw.
4. 10 x von Frey 6 g to each hind paw.
5. 1 x acetone to each hind paw.
6. 10 x von Frey 8 g to each hind paw.
7. 1 x acetone to each hind paw.
8. 1 x acetone to each hind paw.

Withdrawal responses were confirmed by full lifting of the foot from the stimuli. Flinching and partial lifting were ignored. Difference scores were used to quantify pain-like behaviour and were calculated as follows: Difference score = number of paw withdrawals from the injured (ipsilateral) paw minus noumber of paw withdrawals from uninjured (contralateral) paw.

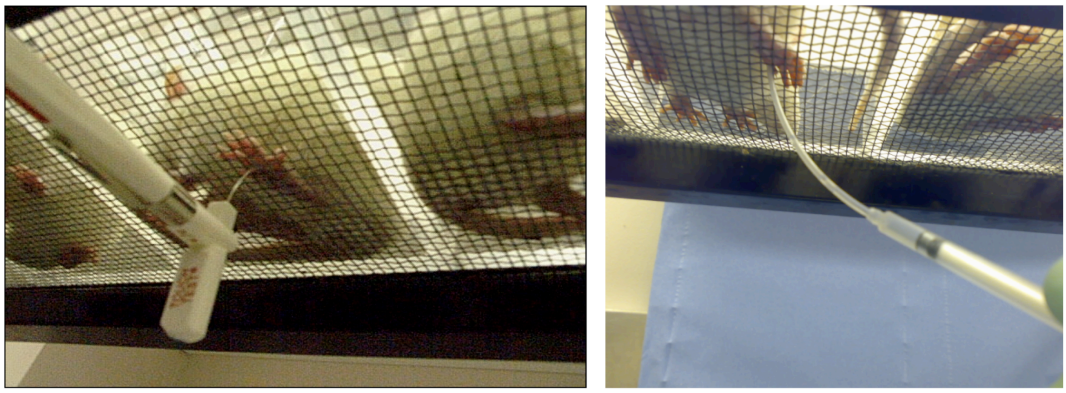


Figure 2.5 Testing of pain-like behaviour. Von Frey filaments of varying forces (left) and acetone (right) applied to hind paw.

In order to determine the effect of drugs at the spinal level on pain-like behaviour, 14 days post SNL, rats were first lightly anaesthetised on 2 % isofluorane in a mixture of nitrous oxide (50 % v/v) and oxygen (50 % v/v), after which the were disinfected and lightly shaved across their back. A 0.5 ml insulin syringe (BD Micro-Fine^{TM}) was used to inject 20 μl of drug solution through the skin, into the L5 – L6 vertebral interspace, after which the rats were allowed to recover. The drug regimen was blinded until the analysis was complete. After all behavioural studies, rats were overdosed on a rising concentration of CO_2, after which, death was ensured by cervical dislocation of the neck.

2.4.3 In vivo electrophysiology recordings from spinal cord neurones

All neuronal recordings were taken from the side ipsilateral to the nerve ligations. See 2.2.

2.5 Carrageenan-induced inflammation model

This model is an adaptation on the formalin test, yet it presents with differing features which have been well characterised (Kayser and Guilbaud, 1987; Stanfa et al., 1992). With formalin-induced inflammation, behavioural hypersensitivity presents as spontaneous licking, biting, lifting and flinching of the injected hind paw, which peaks within 1 hr (Dubuisson and Dennis, 1977). Behavioural hypersensitivity due to carrageenan-induced inflammation however is more apparent when assessing withdrawal thresholds to noxious mechanical and thermal stimuli i.e. the thresholds are lowered thus indicating mechanical and thermal hyperalgesia. Behavioural hypersensitivity in this model peaks within 3 hr, yet mechanical hypersensitivity is still present at 20 hr even though thermal hypersensitivity has subsided (Hedo et al., 1999).

2.5.1 In vivo electrophysiology recordings from spinal cord neurones

See 2.2. To monitor stimulus evoked neuronal activity as a result of carrageenan-induced inflammation, a 2 % (w/v) solution made from carrageenan powder (Sigma Aldrich, USA) was intradermally injected into the hind paw ipsilateral to the WDR neurone being recorded, using a 0.5 ml insulin syringe (BD Micro-Fine^{TM}). All WDR neurones responded to both innocuous and noxious stimuli and all studies were performed at t = 3 hr post carrageenan injection i.e. at the peak of behavioural hypersensitivity.

2.6 Immunohistochemistry (SNL rats)

2.6.1 Perfusion and tissue preparation

14 days post SNL, rats were terminally anaesthetised with 1 ml (200 mg) pentobarbitone sodium intraperitoneally (i.p.) The thoracic cage was then opened and the heart was exposed and freed from the pericardium. Animals were then transcardially perfused with 200 ml saline (0.9 % w/v NaCl solution) with added heparin solution (LEO laboratories UK, 1000 IU of heparin per l of saline. This was followed by 300 ml per rat of 4 % w/v paraformaldehyde (VWR, UK) solution in 0.1 M phosphate buffer (PhB) mixed with 0.15 % picric acid (Sigma Aldrich, UK) to 'strengthen' the fix. A laminectomy was performed similar to that for in vivo electrophysiology (see section 2.2.1), except much more vertebrae were removed, starting from the sacral region, all the way up to the cervical region. DRG from L4, L5 and L6 were removed from both the ipsilateral and contralateral side of the ligations. The lumbar/sacral region (L3 – S2) of the spinal cord was also removed. DRG were post-fixed for 2 hours and spinal cord overnight, before they were transferred to a cryoprotectant solution containing 30 % w/v sucrose in 0.1 M PhB and 0.01 % w/v sodium azide (Sigma Aldrich, UK) for a minimum of 24 hours. All tissues were embedded in optimal cutting temperature (OCT) compound and rapidly frozen on liquid nitrogen before being stored at –80 ºC prior to cutting. Transverse sections were cut using a cryostat (20 μm) and mounted on Superfrost® Plus slides. These slides were then stored at –20 ºC in a cryoprotectant solution comprising 40 % v/v phosphate buffered saline (PBS), 30 % v/v ethylene glycerol and 30 % v/v glycerol.

2.6.2 Standard staining

Slides containing tissue sections were washed in PBS six times for 5 min per wash to remove the cryoprotectant solution. A mixture of 10 % v/v normal donkey serum in PBS plus 0.2 % v/v triton X-100 (Sigma Aldrich, UK) and 0.1% w/v sodium azide was applied to slides for 30 min to minimise non-specific binding. The donkey serum was removed and primary antibodies in PBS plus 0.2 % v/v triton X-100 and 0.1 % w/v sodium azide were added to the slides overnight. The following day, the slides

were washed in PBS three times for 5 min per wash to remove excess primary antibody. The secondary fluorescent antibody was then applied in the same way as the primary antibody and left in the dark for 3 hr. The slides were then washed in PBS 3 times for 5 min per wash to remove excess secondary antibody. A small amount of Vectashield® solution was then placed onto each slide and a cover slip was carefully positioned over the solution and tissue. Finally, the cover slip was sealed onto the slide with nail varnish.

2.6.3 Tyramide signal amplification (for phospho p70S6K)

Slides containing tissue sections were immersed overnight in antigen unmasking solution (Vector laboratories Inc, USA) 1:100 in PBS plus 0.2 % v/v triton X-100 and 0.1 % w/v sodium azide. The next day, the slides, still immersed in solution were heated on high power (800 W) for 1 min 15 s. After a cooling period of 30 min, the slides were washed in PBS three times for 5 min per wash to remove the unmasking solution. A solution of 0.3 % v/v H_2O_2 was then applied to the slides for 20 min to remove all endogenous peroxidase activity and therefore decrease the amount of background signal due to the peroxidase-conjugated antibodies. It also helps to activate the tyramide (Polak and Van Noorden, 2003).

The slides were then washed in PBS three times for 5 min per wash to remove H_2O_2. A mixture of 10 % v/v normal donkey serum in PBS plus 0.2 % v/v triton X-100 (Sigma Aldrich, UK) and 0.1% w/v sodium azide was applied to slides for 30 min to minimise non-specific binding. The donkey serum was removed and primary antibody (rabbit phospho p70S6K Thr389, New England Biolabs, UK) at an optimal concentration of 1:50 in PBS plus 0.2 % v/v triton X-100 and 0.1 % w/v sodium azide was added to the slides overnight.

The following day, the slides were washed in PBS three times for 5 min per wash to remove excess primary antibody. The secondary biotinylated antibody (goat anti-rabbit biotin, Vector laboratories, USA) was then applied in the same way as the primary antibody at a concentration of 1:400 for 1.5 hr. This biotinylated secondary antibody recognises the primary rabbit antibody and is the first stage of the amplification process. The slides were washed in PBS three times for 5 min per

wash to remove excess biotinylated antibody. Next, slides were incubated with avidin biotin labelled complex (ABC Elite, Vector Lab, USA) (1:250 PBS Vectastain solution A plus 1:250 PBS Vectastain solution B) for 30 min followed by signal amplification step with biotinylated tyramide solution (1:75 PBS for 10 min, Perkin Elmer, USA). The avidin and biotin react together, forming large, highly labelled complexes. The proportion of avidin to labelled biotin is such that some binding sites on the avidin are left free to attach to the biotin on the secondary biotinylated antibody (Polak and Van Noorden, 2003). The slides were washed in PBS three times for 5 min per wash to remove the reaction components and a fluorescent extra-avidin fluorescein isothiocyanate (FITC) was used to bind to the biotin attached to the tyramide.

2.6.4 Analysis

Sections were viewed under an Axioplan 2 Imaging microscope (Imaging associates, UK) fitted with 10 x, 20 x and a 40 x Plan-Neofluoro objectives (Zeiss, UK). Images were taken using an Axioplan digital camera (Zeiss, UK) and AxioVision software (Imaging Associates, UK). Image analysis was performed with the National Institutes of Health (NIH) software Image J version 1.38 (USA). Post-acquisition processing was performed with Adobe Photoshop version 7.0 (USA). For statistical analysis, a minimum of 3 sections per group were chosen at random.

2.7 Statistical analysis

N.B. All statistical analyses were completed using GraphPad Prism version 4.0c (USA). See individual chapters for specific details of tests employed.

3 Rapamycin-sensitive pathways under physiological conditions

3.1 Introduction

Apart from an important role in neuronal development, synaptic transmission and plastic processes, mTOR and its associated pathways are important in a wide range of physiological processes including cell growth, proliferation and animal size (Hay and Sonenberg, 2004; Sarbassov dos et al., 2005; Jaworski and Sheng, 2006; Swiech et al., 2008). In light of this, it was of great importance to understand what effects if any, inhibiting neural pathways with rapamycin, would have on neuronal output in naive animals over a full range of innocuous and noxious stimuli. Not only is this a useful strategy in determining safe and effective doses of rapamycin, but it also means predictions can be made about potential effects in a pathophysiological state such as a persistent pain-like state.

3.1.1 Toxicity

A 2 d treatment with rapamycin (200 nM) has been found not to show any toxic effects on cultured cells even with this direct contact with the cells. In contrast, 2 widely used protein synthesis inhibitors- anisomycin and cyclohexamide appear to be toxic to nerve-muscle cultures when treated for the same amount of time (Je et al., 2005). This dose of rapamycin used was effective in attenuating protein synthesis induced by neurotrophin 3 (NT3). This coupled with the fact that the immunosuppressant rapamycin, is still used as an immunosuppressant after kidney transplant and is now used in clinical cancer research (Cohen, 2002; Faivre et al., 2006) confirms that at the right dose, toxicity is not a major issue, although side effects connected with suppression of the immune system still need to be taken into account.

3.1.2 Reversibility

Rapamycin-sensitive pathways have been shown to be important during the induction phase of LLTP in hippocampal slices. However, when rapamycin (which inhibits LLTP if administered during the induction phase) is transiently added to hippocampal slices for 40 min and the LLTP-inducing tetanisation paradigm is delivered after washing out the inhibitor, LLTP can still be elicited (Cammalleri et al., 2003). This supports the notion that the action of rapamycin is indeed reversible.

3.1.3 Activity-dependency

In many cases, the effects that rapamycin exerts are only apparent when there is a sufficient stimulus that engages the appropriate pathways. For example, treating synaptosomes for 10 min with 5-HT has been shown to induce a decrease in the phosphorylation (and therefore inactivation) of the elongation factor eEF2. In the presence of rapamycin, 5-HT no longer induced this decrease. However, rapamycin in the absence of 5-HT had no such effect on eEF2 (Carroll et al., 2004). Furthermore, whilst rapamycin will successfully attenuate the amount of LLTP achieved with the required tetanisation stimulus, rapamycin alone, in the absence of a tetanus, will have no effect on basal synaptic transmission suggesting that rapamycin-sensitive pathways are much more important in longer lasting forms of plasticity (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005). Also, when nerve-muscle cocultures are incubated with the growth factor NT3 and rapamycin together for 2 d, the long-term effects of NT3 on synapses (new protein synthesis) are abolished. However, the effect of chronic NT3 on potentiating acute spontaneous synaptic transmission is unchanged by rapamycin (Je et al., 2005).

In vivo behavioural studies have shown that whilst local rapamycin injections are successful in reducing the amount of secondary or referred mechanical hyperalgesia induced by capsaicin as well as mechanical hypersensitivity in a rat model of nerve injury-induced chronic pain, they are ineffective in attenuating acute nociceptive thresholds (Jimenez-Diaz et al., 2008). Thus, rapamycin-sensitive

pathways can potentially be continually upregulated and are dependent upon specific types of stimuli. The aim of these studies was to identify how true this was using in vivo electrophysiology to study neuronal responses to rapamycin and the ester form of rapamycin, cell cycle inhibitor (CCI)-779 (Temsirolimus, USA) administration.

3.2 Methods

3.2.1 Rapamycin compounds

Two different forms of rapamycin have been used throughout these studies: rapamycin (sirolimus, LC laboratories, USA) which was initially dissolved in 100 % dimethyl sulphoxide (DMSO) and CCI-779, which was dissolved in saline with the aid of a sonicator. Once rapamycin was dissolved in DMSO, the mixture was then dissolved in saline to obtain the appropriate concentrations. The maximum concentration of DMSO never exceeded 25 % v/v. There was a strong preference towards using CCI-779, due to the fact that it has a much more improved solubility in water compared with the non-ester form of rapamycin and therefore making it more ideal for in vivo studies (Lee et al., 2006). In addition, 100 % DMSO has been shown to cause short-term (2 min) scratching behaviour when injected i.t. into mice (Welch et al., 1995) and DMSO concentrations as low as 9 % v/v have been shown to block C-fibre responses in the cat when directly applied to the nerves (Evans et al., 1993). The non-ester form of rapamycin was obtained before obtaining CCI-779 and, due to constraints on time it was not possible to use CCI-779 throughout these studies. Therefore some studies involved the non-ester form of rapamycin, which is referred to as rapamycin, and others have involved CCI-779.

3.2.2 In vivo electrophysiology setup

See 2.2. After obtaining a stable baseline, dose-response data was generated using rapamycin. The maximal vehicle concentration- 25 % v/v DMSO was applied and the effects followed before administering 25 nM (1.14 ng in 50 μl saline/DMSO), 125 nM (5.72 ng in 50 μl saline/DMSO) and 250 nM (11.43 ng in 50 μl saline/DMSO) rapamycin in a cumulative fashion i.t. to the exposed spinal cord. Electrical stimuli

and 'natural' stimuli were applied every 20 min, for 1 hr, before the next dose of drug was added. For CCI-779, only the top dose of 250 nM (12.88 ng in 50 μl saline) was used and this was followed for 2 hr. This was compared with an effective 4.7 mM (62.35 μg in 50 μl saline/DMSO) dose of anisomycin (Sigma Aldrich, UK) (Jimenez-Diaz et al., 2008), a general translation inhibitor that acts by inhibiting peptidyl transferase activity in eukaryoyte ribosomes. Anisomycin was first dissolved in 100 % DMSO before diluting with saline to obtain a concentration of 4.7 mM with a DMSO content of 10 % v/v.

3.2.3 Statistical analysis

Electrophysiological raw data are presented as mean number of spikes ± SEM. When comparing baseline neuronal responses to maximum change (positive or negative) neuronal responses after administration of DMSO, student's t-tests were used to compare differences in Aβ-, Aδ- and C-fibre firing, post-discharge, input, wind up and brush. Two way analysis of variance (two way ANOVA) of maximum change data with repeated measures and Bonferroni's post-tests were used to determine significance between groups for natural stimuli i.e. graded mechanical and thermal stimuli. For rapamycin, CCI-779 and anisomycin, one way ANOVA of maximum change data with repeated measures Dunnett's post-test comparisons to control were used to determine significant changes for electrical stimuli as well as brush and pin (control = 0 min for CCI-779 and anisomycin and 25 % DMSO for rapamycin). Two way ANOVA of maximum change data with repeated measures and Bonferroni's post-tests were used to determine significant changes for graded natural stimuli i.e. graded mechanical and thermal stimuli (*$P<0.05$; **$P<0.01$ ***$P<0.001$).

3.3 Results

3.3.1 DMSO has no significant effect on baseline neuronal responses

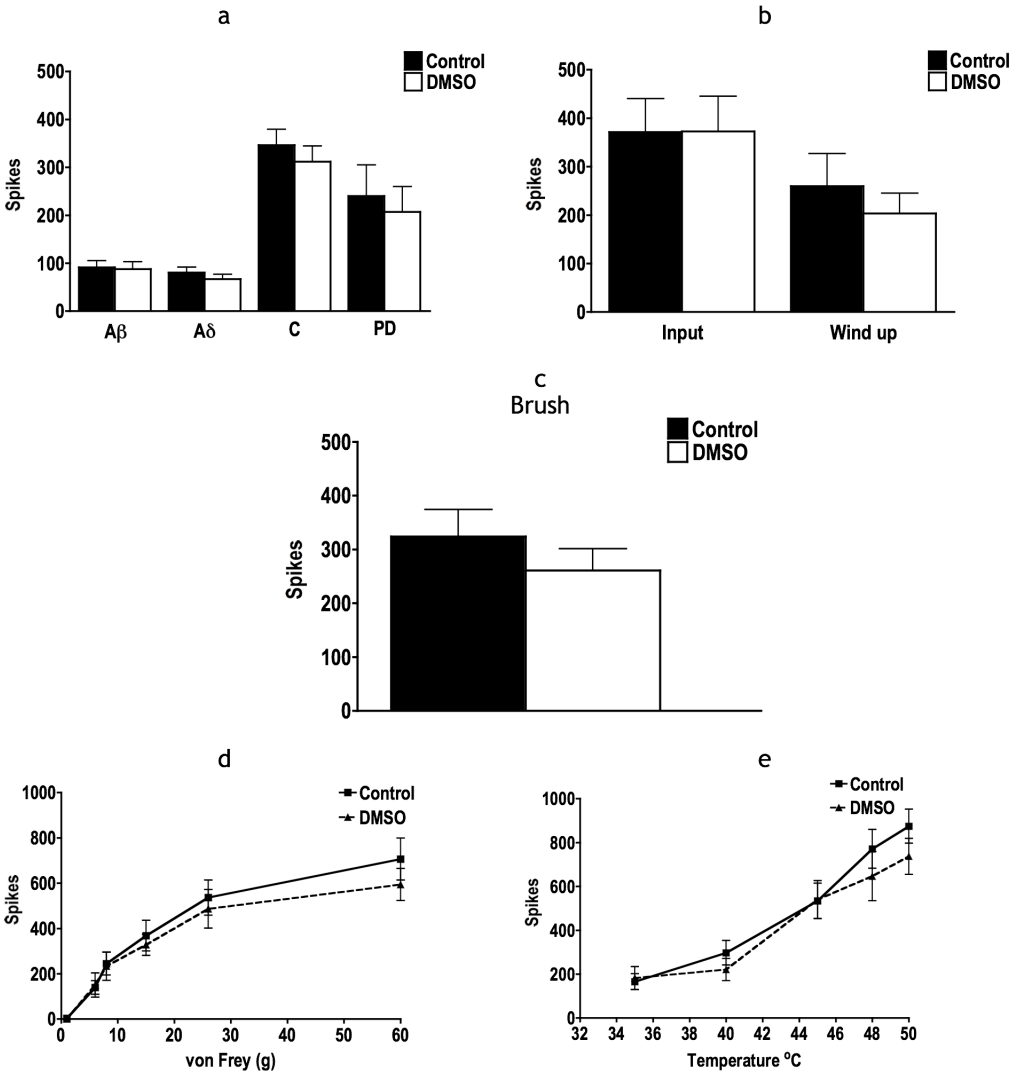


Figure 3.1 Effects of 25 % DMSO on neuronal responses. (a) There were no significant effects of DMSO on pre-drug control responses for electrically evoked Aβ, Aδ and C-fibre-mediated transmission as well as post-discharge (PD); (b) electrically induced input and wind up (number of spikes after a train of 16 pulses); (c) brush (number of spikes during a 10 s stimulus); as well as (d) graded mechanically evoked (number of spikes during a 10 s stimulus); and (e) graded thermally evoked responses (number of spikes during a 10 s stimulus, see 2.2 and 3.2.2 for methods). For all data sets, pre-drug control n = 10, DMSO n = 8.

3.3.2 Rapamycin inhibits electrically evoked neuronal responses

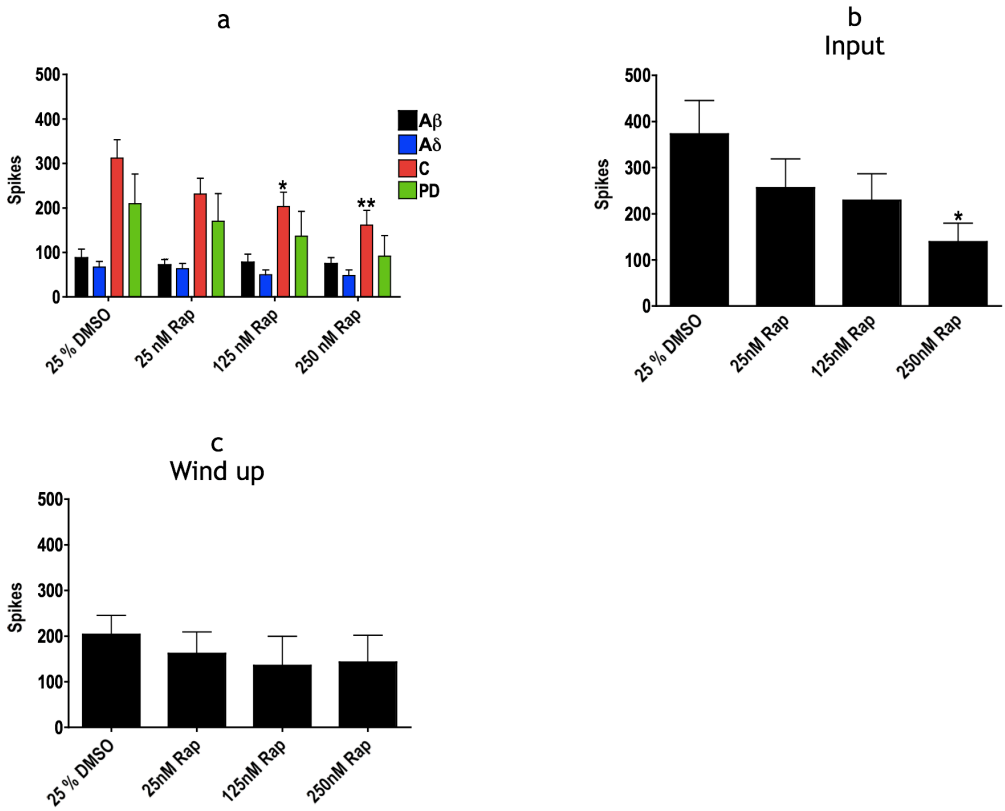


Figure 3.2 Effects of rapamycin on electrically evoked neuronal responses. (a) C-fibre-mediated transmission onto WDR neurones was significantly inhibited by 125 and 250 nM rapamycin (Rap) compared to 25 % DMSO from 312 ± 33 to 203 ± 32 and 161 ± 30 spikes respectively. Aβ- and Aδ-fibre mediated transmission were unaffected as was WDR neurone post-discharge (PD). (b) Input was also significantly reduced with 250 nM rapamycin compared to 25 % DMSO from 373 ± 72 to 139 ± 40 spikes. (c) Wind up however was unaffected by rapamycin (see figure 3.2.2 for methods) For all data sets, spikes = number of spikes after a train of 16 pulses; n = 10 except 250 nM Rap where n = 9 (*P<0.05; **P<0.01).

3.3.3 Rapamycin inhibits mechanically evoked neuronal responses

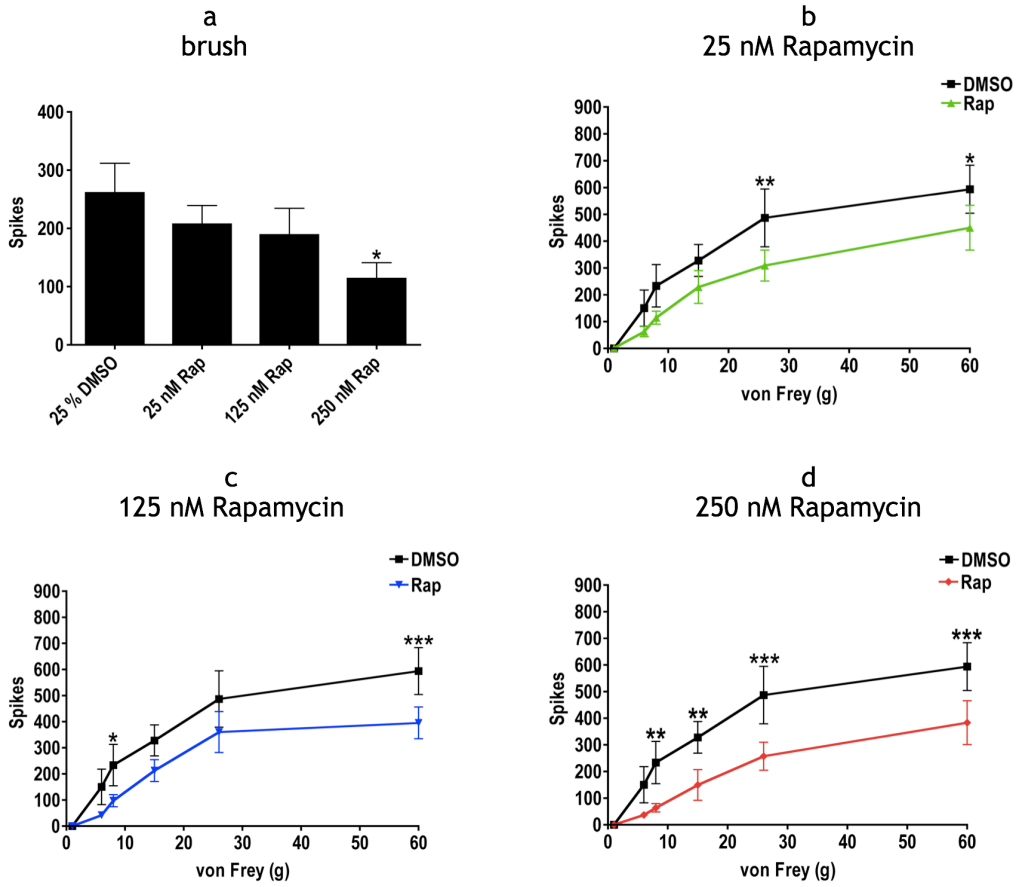


Figure 3.3 Effects of rapamycin on mechanically evoked neuronal responses. (a) Rapamycin (Rap) inhibited dynamic brush responses at 250 nM compared to 25 % DMSO (black) from 261 ± 40 to 114 ± 25 spikes. (b) 25 nM Rap (green) significantly inhibited responses to 26 and 60 g compared to 25 % DMSO (black) from 487 ± 86 to 309 ± 58 and 594 ± 71 to 450 ± 83 spikes respectively. (c) 125 nM Rap (blue) significantly inhibited responses to 8 and 60 g compared to 25 % DMSO (black) from 234 ± 63 to 97 ± 23 and 594 ± 71 to 395 ± 61 spikes respectively. (d) 250 nM Rap (red) significantly inhibited responses to 8, 15, 26 and 60 g compared to 25 % DMSO (black) from 234 ± 63 to 64 ± 14; 328 ± 47 to 149 ± 52; 487 ± 85 to 257 ± 50 and 594 ± 71 to 383 ± 74 spikes respectively. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 10 except 250 nM Rap, where n = 9 (*P<0.05; **P<0.01; ***P<0.001).

3.3.4 Rapamycin inhibits graded thermally evoked neuronal responses

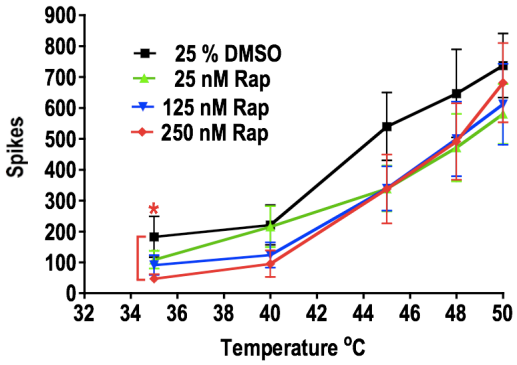


Figure 3.4 Effects of rapamycin on graded thermally evoked neuronal responses. 250 nM rapamycin (Rap) was only effective in attenuating responses evoked by 35 °C from 183 ± 66 (25 % DMSO) to 47 ± 15 spikes. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 10 except 250 nM rapamycin, where n = 9. (*P<0.05).

The data so far suggests that rapamycin has dose-dependent effects on stimulus evoked responses of WDR neurones. Specifically, rapamycin dose-dependently inhibited C-fibre mediated transmission to WDR neurones, non-potentiated input, dynamic mechanical stimuli (bush) and punctate mechanical stimuli including innocuous (≤ 8 g) and noxious (≥ 15 g) von Frey filaments. However, the effects on thermally evoked responses were minimal and only the top dose inhibited responses to the lowest innocuous stimulus (35 °C). It was decided that this dose would also be studied using CCI-779. The effects of this drug were followed for a longer time period of 2 hr, with all comparisons being made to pre-drug control responses (at 0 min). The effects of the global translation inhibitor anisomycin were also studied in the same manner and was used as a positive control to confirm that effects of rapamycin were due to inhibition of translation (Jimenez-Diaz et al., 2008).

3.3.5 CCI-779 has no effect on electrically evoked neuronal responses

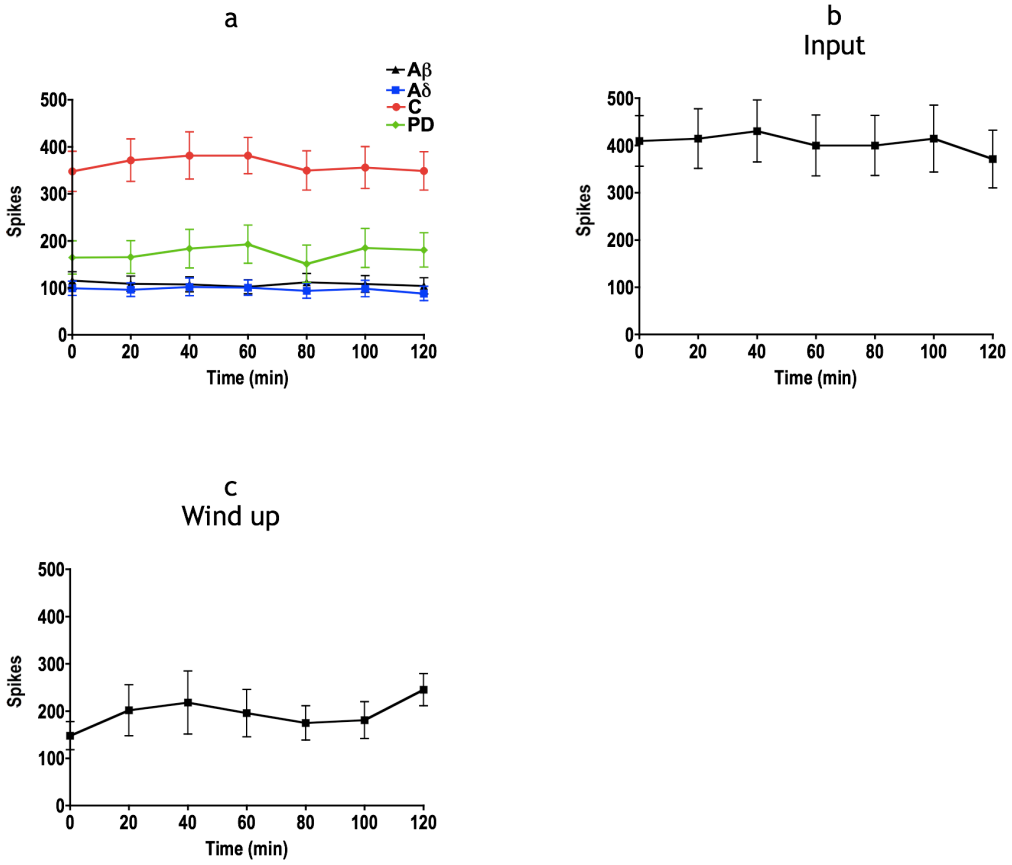


Figure 3.5 Effects of CCI-779 on electrically evoked neuronal responses from naive rats over 2 hr. (a) 250 nM CCI-779 was ineffective on Aβ-, Aδ-, and C-fibre mediated transmission as well as post-discharge (PD) of WDR neurones. (b) Input as well as (c) wind up were also unaffected by CCI-779. For all data sets, spikes = number of spikes after a train of 16 pulses; n = 9, except wind up where n = 8.

3.3.6 CCI-779 has no effect on brush and pinprick evoked neuronal responses

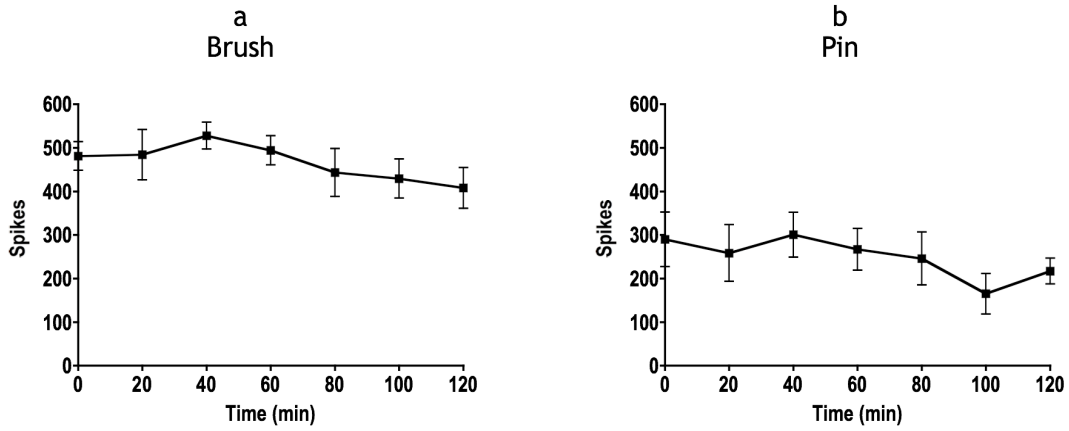


Figure 3.6 Effects of CCI-779 on brush and pinprick evoked neuronal responses from naive rats over 2 hr. 250 nM CCI-779 was ineffective on (a) innocuous, dynamic brush evoked stimuli (n = 9) and (b) noxious punctate pinprick evoked stimuli (n = 5). For all data sets, spikes = number of spikes during a 10 s stimulus.

3.3.7 CCI-779 has selective effects on graded mechanically and thermally evoked neuronal responses

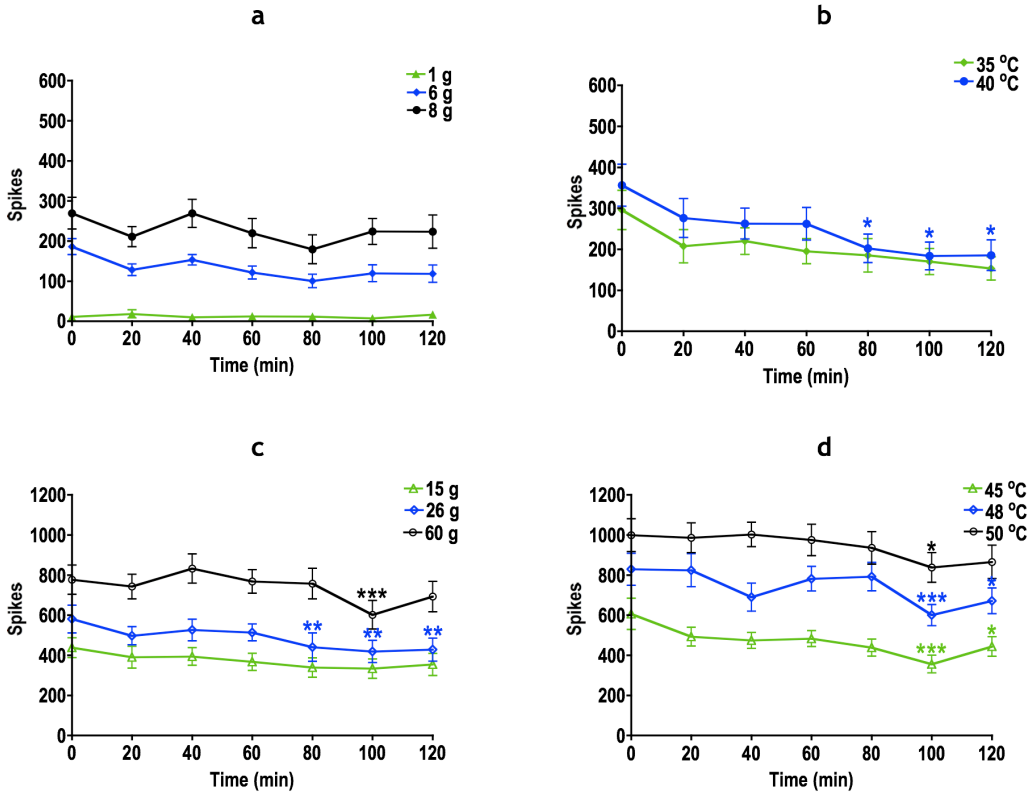


Figure 3.7 Effects of CCI-779 on graded mechanically and thermally evoked neuronal responses from naive rats over 2 hr. (a) CCI-779 has no effects on innocuous mechanically evoked responses. (b) At 80, 100 and 120 min, CCI-779 significantly inhibited innocuous thermally evoked responses to 40 °C from 356 ± 51 (at 0 min) to 202 ± 35; 184 ± 34 and 186 ± 37 spikes respectively. (c) At 80, 100 and 120 min, CCI-779 significantly inhibited noxious mechanically evoked responses to 26 g from 580 ± 69 (at 0 min) to 440 ± 71; 419 ± 55 and 428 ± 57 spikes respectively. Also at 100 min, CCI-779 significantly inhibited noxious mechanically evoked responses to 60 g from 777 ± 73 (at 0 min) to 602 ± 72 spikes. (d) At 100 min, CCI-779 significantly inhibited noxious thermally evoked responses to 45, 48 and 50 °C from 606 ± 78 (at 0 min) to 356 ± 44; 829 ± 80 (at 0 min) to 600 ± 53 and 999 ± 82 (at 0 min) to 838 ± 74 spikes respectively. At 120 min, CCI-779 significantly inhibited noxious thermally evoked responses to 45 and 48 °C from 606 ± 78 at (at 0 min) to 444 ± 49 and 829 ± 80 (at 0 min) to 671 ± 65 spikes respectively. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 9 (*P<0.05; **P<0.01; ***P<0.001).

3.3.8 Anisomycin has no effect on electrically evoked neuronal responses

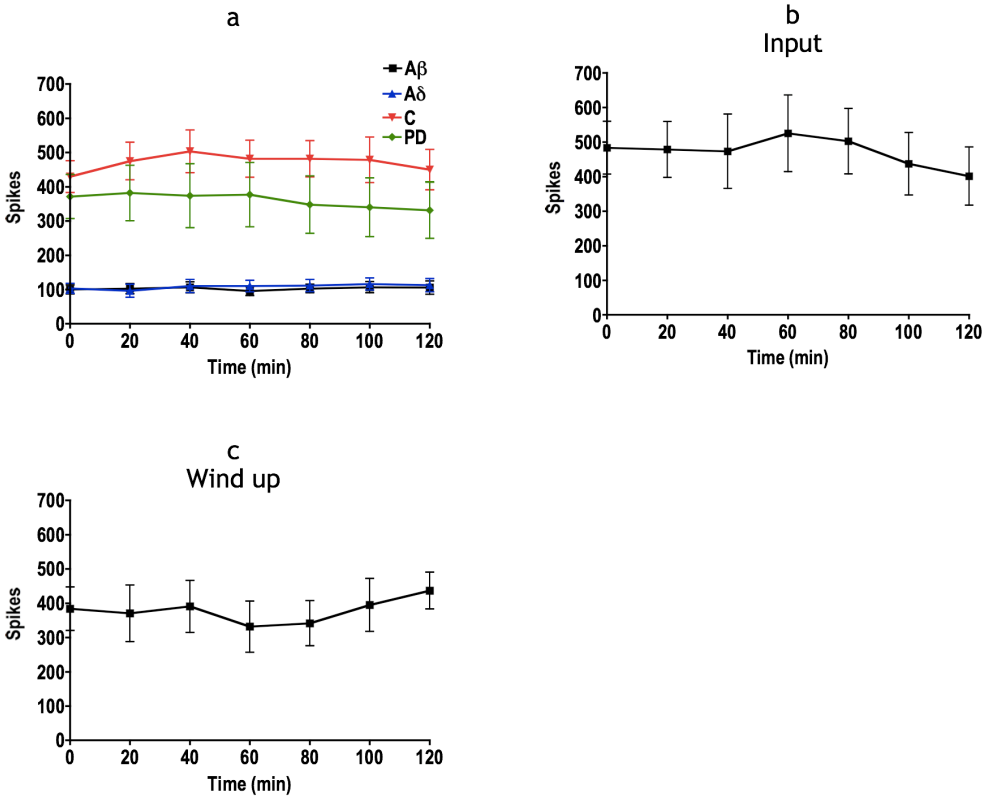


Figure 3.8 Effects of anisomycin on electrically evoked neuronal responses from naive rats over 2 hr. (a) Anisomycin was ineffective on Aβ-, Aδ-, and C-fibre mediated transmission and post-discharge (PD) of WDR neurones. (b) Input as well as (c) wind up were also unaffected by anisomycin. For all data sets, spikes = number of spikes after a train of 16 pulses; n = 12.

3.3.9 Anisomycin has no effect on brush and pinprick evoked neuronal responses

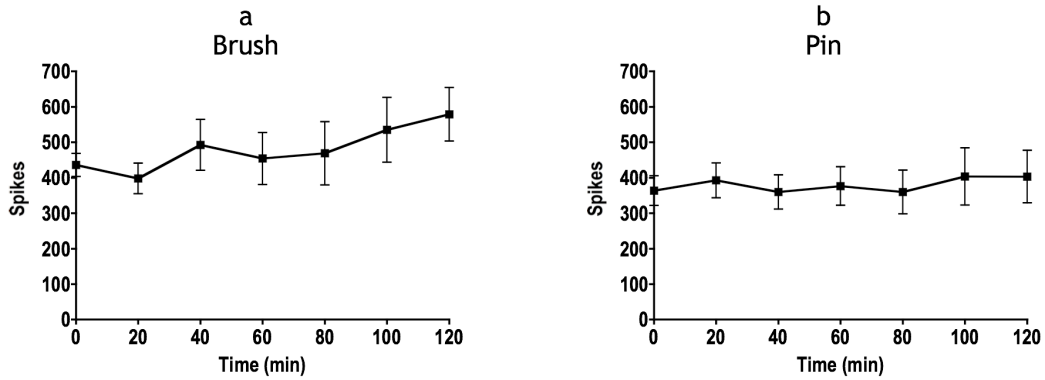


Figure 3.9 Effects of anisomycin on brush and pinprick evoked neuronal responses from naive rats over 2 hr. Anisomycin was ineffective on (a) innocuous, dynamic brush evoked stimuli and (b) noxious punctate pinprick evoked stimuli. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 12.

3.3.10 Anisomycin has no effect on graded mechanically or thermally evoked neuronal responses

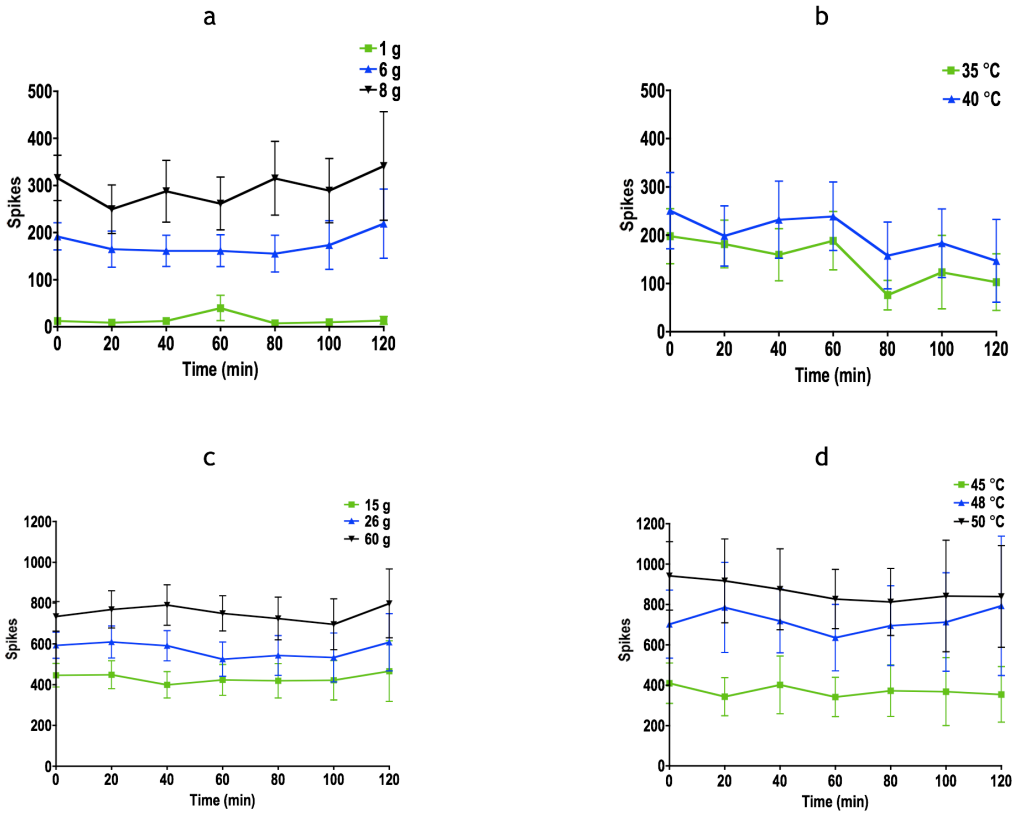


Figure 3.10 Effects of anisomycin on graded mechanically and thermally evoked neuronal responses from naive rats over 2 hr. Anisomycin was ineffective against innocuous graded mechanical and thermal stimuli (a and b respectively) as well as graded noxious mechanical and thermal stimuli (c and d respectively). For all data sets, spikes = number of spikes during a 10 s stimulus; n = 12.

To summarise, 25 % DMSO had no effect on baseline responses at this concentration (see figure 3.1), which is the maximum concentration achieved when administering the highest dose of rapamycin. When compared to responses evoked after the administration of different doses of rapamycin, it was found that rapamycin inhibited responses to electrically, mechanically and thermally evoked responses (see figures 3.2 - 3.4). These effects were found to be dose-dependent, with a higher degree of significance occurring with the top dose.

CCI-779 also exerted inhibitory effects on stimulus evoked responses of WDR neurones. However, unlike the non-ester form of rapamycin, there were no inhibitory effects on electrically evoked responses (see figure 3.5). Instead, inhibitory actions were restricted to responses evoked by 26 and 60 g von Frey stimuli and 40, 45, 48 and 50 °C thermal stimuli (see figure 3.7). Furthermore, unlike the non-ester form of rapamycin, these actions were exerted in the second hr of testing. In contrast, the general translation inhibitor anisomycin was ineffective against all measures under these conditions (figures 3.8 – 3.10).

3.4 Discussion

The aim of these studies was to identify the importance of rapamycin-sensitive pathways under physiological conditions. 2 different drugs were used in order to achieve this. Firstly, the non-ester form of rapamycin (rapamycin) and secondly the ester form of rapamycin (CCI-779). These are 2 drugs which differ in their water solubility, with CCI-779 being much more soluble in water.

Dose-response studies were carried out using rapamycin. Dose-dependent inhibition of nociceptive specific C-fibre-mediated transmission and presynaptic input indicates that rapamycin-sensitive pathways are important in transmission from C-fibres to the spinal cord. Presumably, this inhibition of C-fibre activity is responsible for the accompanying inhibition of mechanically evoked responses. The comparatively minimal effects on thermally evoked responses (250 nM only inhibits responses to 35 °C) reveals a higher degree of selectivity of rapamycin for mechanically evoked rather than thermally evoked responses.

Although establishing that rapamycin exerted maximal effects within the first hr of administration, this was not the case for CCI-779. With CCI-779, there were no effects on electrically evoked responses and all other effects on mechanically and thermally evoked responses occurred after the first hr of drug administration. So why does this happen?

Firstly, the studies with rapamycin involve the cumulative addition of 3 doses of the drug: 25, 125 and 250 nM. Although all maximal drug effects were within the first hr of administration, a 250 nM dose on top of previous 25 and 125 nM doses would amount to a potential maximal final dose of 400 nM depending on clearance rates and drug half-life. In support of this is the fact that rapamycin and it's associated analogues have a half life of >30 min (Wyeth Research, data unpublished), suggesting that if it were not cleared within the 1 hr time frame that was provided for each dose, then dose accumulation could easily occur. However, it should be noted that mechanical von Frey stimulus evoked responses were affected from the lowest dose of rapamycin (see figure 3.3).

Secondly, although the solubility of CCI-779 in water is much better than that of rapamycin, it may be less soluble in saline. Although this could be the case because CCI-779 had to be sonicated in order to achieve solubilisation, the fact that solubilisation was achieved should mean that this is not a factor. Nevertheless, it may be that DMSO achieves slight but significantly improved solubilisation. This may explain why inhibitory effects were not seen with electrically evoked responses and why the effects were not seen until 1 hr after CCI-779 administration. Interestingly, the effects seen with rapamycin and CCI-779 do not match the effects seen with anisomycin. However, this may be purely due to a difference in potency i.e. a 250 nM dose of rapamycin or CCI-779 may be much more efficacious than a 4.7 mM dose of anisomycin.

These studies demonstrate that rapamycin-sensitive pathways are at least partially important under physiological conditions. This is not surprising due to the involvement of mTOR in neuronal development and synaptic plasticity (Swiech et al., 2008). It does however contradict the studies, which report that rapamycin has no effect on basal synaptic activity (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005; Je et al., 2005). However, it is important to note that all these previous studies were performed in vitro on isolated tissue where important physiological processes are attenuated or completely absent, networks of neurones are disrupted and afferent sensory evoked activity cannot be studied. In this chapter, spinal neurones were activated in vivo by a range of innocuous and noxious suprathreshold stimuli applied to peripheral sites.

4 Rapamycin-sensitive pathways and pathophysiological conditions: formalin-induced inflammation

4.1 Introduction

The formalin test was first presented by Dubuisson and Dennis in 1977 (Dubuisson and Dennis, 1977). The test results in two key features: biphasic ongoing neuronal hyperexcitability and behavioural hypersensitivity. The first phase occurs immediately after formalin injection and lasts for 5 – 10 min. After a small pause in activity (typically 5 - 10 min duration), a more prolonged and slowly developing and longer lasting second phase occurs that typically lasts >30 min after its peak. These features are now commonly used as markers of analgesic drug efficacy (Tjolsen et al., 1992; Green et al., 2000).

4.1.1 Formalin-induced inflammation and upstream regulators of mTOR

Although the importance of rapamycin-sensitive pathways in persistent pain-like states has not been extensively studied, there have been reports focussing on the importance of upstream regulators of mTOR in formalin-induced inflammation. The role of phosphorylated CaMKII and ERK are two such regulators and these proteins have been shown to either engage rapamycin-sensitive pathways or synergise with them, leading to protein translation (Gelinas et al., 2007; Kelly et al., 2007; Tsokas et al., 2007). The levels of these proteins in the spinal cord have been assessed by choi et al. In their study, they found via a combination of Western Blots and immunohistochemistry, that intraplantar injection of formalin solution caused an increase in phosphorylated CaMKII and ERK that was restricted mainly to lamina I and II of the spinal cord dorsal horn. Further support for the importance of these regulators in formalin-induced inflammation was the fact that the ERK inhibitor PD988059 and the CaMKII inhibitor KN-93 administered 20 min prior to formalin administration inhibited the second phase of the formalin response (Choi et al., 2006).

Also upstream of mTOR is PI3K. A recent study by Pezet et al. has highlighted the importance of PI3K in the establishment of central sensitisation. In this study, in vivo electrophysiology was used to show that the PI3K inhibitor LY294002 dose-dependently attenuated wind up. The significance of this effect was confirmed behaviourally as it also attenuated the second phase of the formalin response when administered i.t. 15 min prior to formalin injection into the hind paw. Furthermore, immunohistochemical studies revealed that administration of LY294002 i.t. prior to formalin injection resulted in a decrease in phosphorylation of CaMKII and ERK, thereby suggesting that phosphorylation of both ERK and CaMKII induced by peripheral inflammation are PI3K-dependent mechanisms (Pezet et al., 2008). In addition, PI3K has also been shown to engage rapamycin-sensitive pathways (Schratt et al., 2004; Takei et al., 2004; Horwood et al., 2006; Chenal and Pellerin, 2007; Kelly et al., 2007; Tsokas et al., 2007).

Further upstream, at the receptor level, NMDA receptor activation has been implicated in formalin-induced neuronal hyperexcitability (Haley et al., 1990) and is also implicated in activation of rapamycin-sensitive pathways (Gong et al., 2006; Gonzalez-Mejia et al., 2006). The electrophysiological studies by Haley et al., using similar in vivo approaches to those used in this chapter, demonstrated that the selective NMDA receptor antagonist D-2-amino-5-phosphonopentanoate (D-AP5) was effective in reducing formalin-induced neuronal hyperexcitability of the second phase when administered to the exposed spinal cord 40 min to prior to the injection of formalin into the hind paw. These findings have also been confirmed behaviourally, where spinal administration of various NMDA receptor antagonists- D-AP5, memantine, MK801, dextrorphan and dextromethorphan via i.t. cannulae 15 min prior to the formalin injection were effective (to varying degrees) in attenuating the second phase of formalin-induced flinching (Chaplan et al., 1997).

The metabotropic glutamate receptors mGluR1 and mGluR5 are two receptors that are also of importance in formalin-induced behavioural hypersensitivity (Varty et al., 2005) and these receptors are also implicated in activating rapamycin-sensitive pathways (Page et al., 2006; Price et al., 2007). In their studies, Varty et al. administered the mGluR5 antagonists- MPEP (2-methyl-6-(phenylehtynyl) pyridine) and MTEP (3[2-methyl-1,3thiazol-4-yl)ethynyl]pyridine), as well as the mGluR1

antagonist LY456236 i.p. and found that they reduced second phase (and even first phase for top doses of MPEP and MTEP) formalin-induced licking and biting when given prior to the injection of formalin into the hind paw.

At the transmitter level, BDNF has been shown to be important in formalin-induced hypersensitivity (Kerr et al., 1999) and has also been shown to activate rapamycin-sensitive pathways (Takei et al., 2001; Tang et al., 2002; Schratt et al., 2004; Takei et al., 2004). In their studies, Kerr et al. administered NGF via the i.p. route to rats. This is a method that is used to mimic peripheral inflammatory states and increases BDNF levels in sensory neurones. The increase in BDNF can be countered by TrkB-IgG, a BDNF binding protein. 30 min prior to formalin injection into the hind paw, TrkB-IgG was administered via an i.t. cannula to rats that had been pre-treated with NGF 24 hr earlier and this resulted in a significant reduction in the behavioural score of second phase formalin-induced behavioural hypersensitivity compared to rats that were administered saline after NGF pre-treatment.

4.1.2 Formalin-induced inflammation: global transcription and translation

There is also direct evidence for the requirement of RNA and protein synthesis in formalin-induced inflammation (Kim et al., 1998). In this behavioural study, mice were injected i.t. with either actinomycin D, a general inhibitor of transcription or anisomycin, a general inhibitor of translation. Although responses to acute noxious stimuli were unaffected, both drugs significantly decreased the second phase of the formalin response when administered 30 min prior to the formalin injection.

4.1.3 Formalin-induced inflammation and mTOR

The importance of mTOR in formalin-induced inflammation has been studied in mice lacking fragile X mental retardation protein (FMRP), another protein that influences protein translation in neurones (Price et al., 2007). This study demonstrated decreased nociceptive sensitisation in *Fmr1* KO mice. Due to the similarities between FMRP and mTOR in terms of their importance in protein translation, it was hypothesized that rapamycin would only inhibit nociceptive

behaviours induced by formalin in WT and not KO mice. This was indeed the case as it was shown that rapamycin dose-dependently attenuated the second phase of the formalin test when administered spinally (i.t.) or peripherally (intradermally into the hind paw) 15 min prior to formalin injection into the hind paw of WT mice. The aim of this chapter was therefore to use in vivo electrophysiological and behavioural studies in rats to confirm some of these findings, but also to add to these findings by correlating an in vivo electrophysiological study with a behavioural study.

4.2 Methods

4.2.1 In vivo electrophysiology recordings from spinal cord neurones

See 2.3.1. Once a WDR neurone had been found and characterised, a top dose of rapamycin (250 nM or 11.43 ng), vehicle (25 % v/v DMSO) or anisomycin (4.7 mM or 62.35 μg) in 50 μl saline/DMSO was added directly to the exposed spinal cord. After 3 min, 50 μl of 5 % v/v formalin was injected intradermally into the hind paw into the receptive field of the WDR neurone and ongoing neuronal activity as a result of formalin was monitored for 1 hr, after which the experiment was terminated.

4.2.2 Assessment of the effects of rapamycin on pain-like behaviour

See 2.3.2. A 250 μM dose of rapamycin (11.43 μg in 20μl saline/DMSO) or DMSO (25 % v/v) was administered i.t. under anaesthesia via a single lumbar injection either 5 or 20 min prior to formalin injection. This dose was chosen as it had been shown to attenuate capsaicin- and nerve injury-induced behavioural hypersensitivity when injected locally into the hind paw (Jimenez-Diaz et al., 2008).

4.2.3 Statistical analysis

All data are expressed as raw values $\pm$ SEM. Unpaired t-tests were used when comparing neuronal responses for specific characteristics of neurones between drug and vehicle treatment groups except for graded thermally evoked responses where two way ANOVA with Bonferroni's post-tests were used. Unpaired t-tests were also used to compare the area under the curve (AUC) of the two phases of the formalin between the different groups. When specifically looking at the time course of the formalin response, two way ANOVA with repeated measures and Bonferroni's post-tests were used to make comparisons between groups. For in vivo electrophysiology data, first phase = 0 – 10 min and second phase = 20 – 60 min. For behavioural data, first phase = 0 – 10 min and second phase = 15 – 60 min (*$P<0.05$; **$P<0.01$; ***$P<0.01$)

4.3 Results

4.3.1 Neurones selected for DMSO or rapamycin treatment were not significantly different

	DMSO (n = 11)	Rapamycin (n = 9)
Depth (μM)	777 ± 36	790 ± 51
Aβ-fibre threshold (mA)	0.27 ± 0.10	0.23 ± 0.11
C-fibre threshold (mA)	0.78 ± 0.17	0.55 ± 0.13
Aβ-fibre spikes	104 ± 9	104 ± 16
Aδ-fibre spikes	92 ± 18	93 ± 19
C-fibre spikes	310 ± 49	315 ± 39
Post-discharge spikes	82 ± 29	133 ± 39
Input spikes	298 ± 59	292 ± 57
Wind up spikes	130 ± 33	233 ± 68
35 °C spikes	179 ± 52	102 ± 34
40 °C spikes	374 ± 91	263 ± 85
45 °C spikes	618 ± 67	693 ± 62
48 °C spikes	579 ± 104	513 ± 118
50 °C spikes	777 ± 91	888 ± 79

Table 4.1 Characterisation of WDR neurones selected for DMSO or rapamycin treatment prior to formalin injection. Thresholds were determined using the appropriate stimulus-response latency profiles. For electrically evoked responses, a train of 16 pulses at three times C-fibre threshold (0.5 Hz, 2 ms pulse width) was applied to the corresponding receptive field. For thermally evoked responses, a small water jet was applied to the corresponding receptive field for 10 s. All data are expressed as raw mean values ± SEM. There was no significant difference between cells that had the 25 % DMSO treatment prior to formalin injection or those that were treated with rapamycin prior to formalin injection.

4.3.2 Rapamycin attenuates the second phase of the formalin response

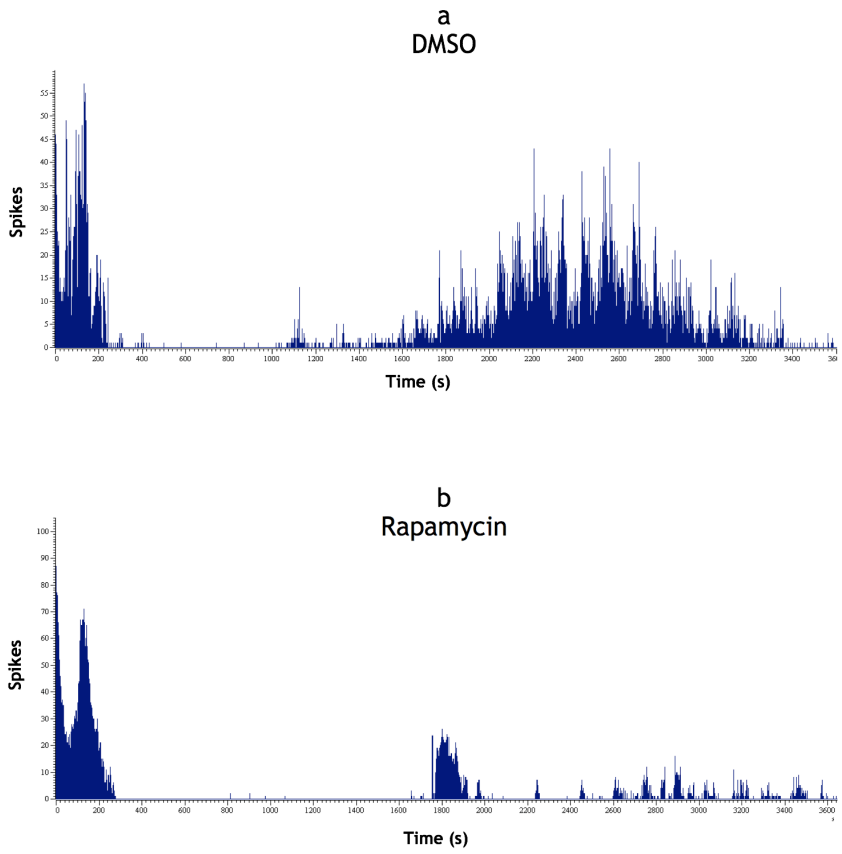


Figure 4.1 Example traces of ongoing neuronal activity due to formalin injection into the hind paw. (a) When DMSO was added 3 min prior to 5 % v/v formalin injection, a classic biphasic response was evoked. The first phase was measured between 0 – 10 min and the second phase was measured between 20 – 60 min. (b) In the presence of rapamycin however, the second phase was attenuated.

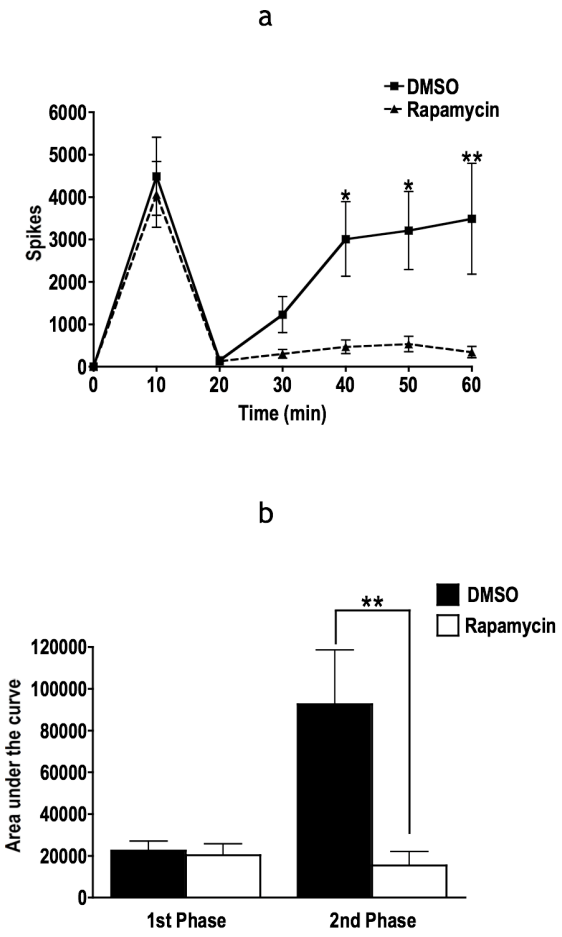


Figure 4.2 Pooled data of responses from WDR neurones during the formalin test. (a) When rapamycin (n = 9) was administered onto the exposed spinal cord 3 min prior to 5 % formalin injection into the hind paw, there was a significant reduction in neuronal activity from 40 - 60 min when compared to DMSO (n = 11) administration from 3,010 $\pm$ 878 to 468 $\pm$ 159; 3,209 $\pm$ 919 to 533 $\pm$ 183 and 3,489 $\pm$ 1,306 to 343 $\pm$ 131 spikes respectively. (b) AUC analysis revealed that overall, rapamycin elicited a significant reduction in the second phase from 92,600 $\pm$ 25,948 to 15,378 $\pm$ 6,623 arbitrary units (*P<0.05; **P<0.01). There were no effects of rapamycin on the first phase of the formalin test.

The formalin test was also repeated in the presence of the general translation inhibitor anisomycin at its effective dose of 4.7 mM compared to 10 % DMSO administered i.t. to the exposed spinal cord 3 min prior to formalin injection into the hind paw.

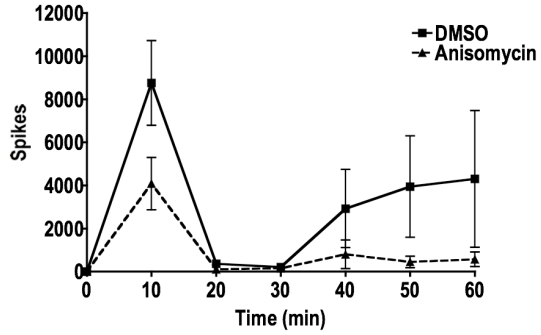
4.3.3 Neurones selected for DMSO or anisomycin treatment were not significantly different

	DMSO (n = 7)	Anisomycin (n = 6)
Depth (μM)	890 ± 85	771 ± 33
Aβ-fibre threshold (mA)	0.70 ± 0.04	0.53 ± 0.11
C-fibre threshold (mA)	1.73± 0.24	1.64 ± 0.16
Aβ-fibre spikes	224 ± 40	176 ± 29
Aδ-fibre spikes	189 ± 24	145 ± 26
C-fibre spikes	597 ± 79	432 ± 33
Post-discharge spikes	755 ± 113	549 ± 119
Input spikes	576 ± 112	640 ± 128
Wind up spikes	752 ± 86	486 ± 103
35 °C spikes	160 ± 50	261 ± 98
40 °C spikes	219 ± 49	351 ± 93
45 °C spikes	626 ± 148	544 ± 104
48 °C spikes	970 ± 145	904 ± 79
50 °C spikes	1409 ± 103	1266 ± 97

Table 4.2 Characterisation of WDR neurones selected for DMSO or anisomycin treatment prior to formalin injection. Thresholds were determined using the appropriate stimulus-response latency profiles. For electrically evoked responses, a train of 16 pulses at three times C-fibre threshold (0.5 Hz, 2 ms pulse width) was applied to the corresponding hind paw. For thermally evoked responses, a small water jet was applied to the corresponding hind paw for 10 s. All data are expressed as raw mean values ± SEM. There were no significant differences between cells that had the 10 % DMSO treatment prior to formalin injection or those that had the anisomycin injection prior to formalin injection.

4.3.4 Anisomycin attenuates the second phase of the formalin response

a



b

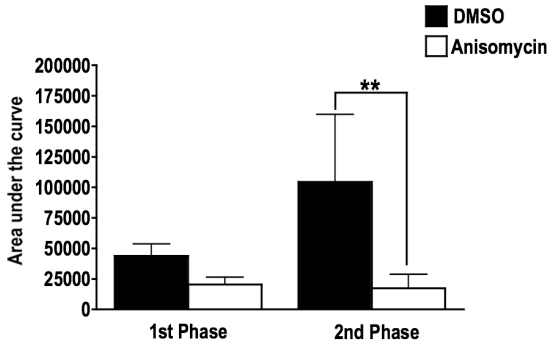


Figure 4.3 Effects of anisomycin on the formalin test. (a) When anisomycin (n = 6) was administered onto the exposed spinal cord 3 min prior to 5 % v/v formalin injection into the hind paw, there was strong trend for reduction in neuronal activity from 10 - 30 min compared to DMSO (n = 7) administration, although there was no significance. (b) AUC analysis however, revealed that overall, anisomycin elicited a significant reduction in the second phase from 104,500 $\pm$ 55,370 to 17,410 $\pm$ 11,360 arbitrary units (*P<0.05). There was no significant effect of anisomycin on the first phase of the formalin test.

Rapamycin was then tested for its effects behaviourally. However, because the rats were not fully conscious by 3 min after anaesthesia, this recovery period was extended to 5 min.

4.3.5 Rapamycin is ineffective against formalin-induced behavioural hypersensitivity when administered 5 min before formalin

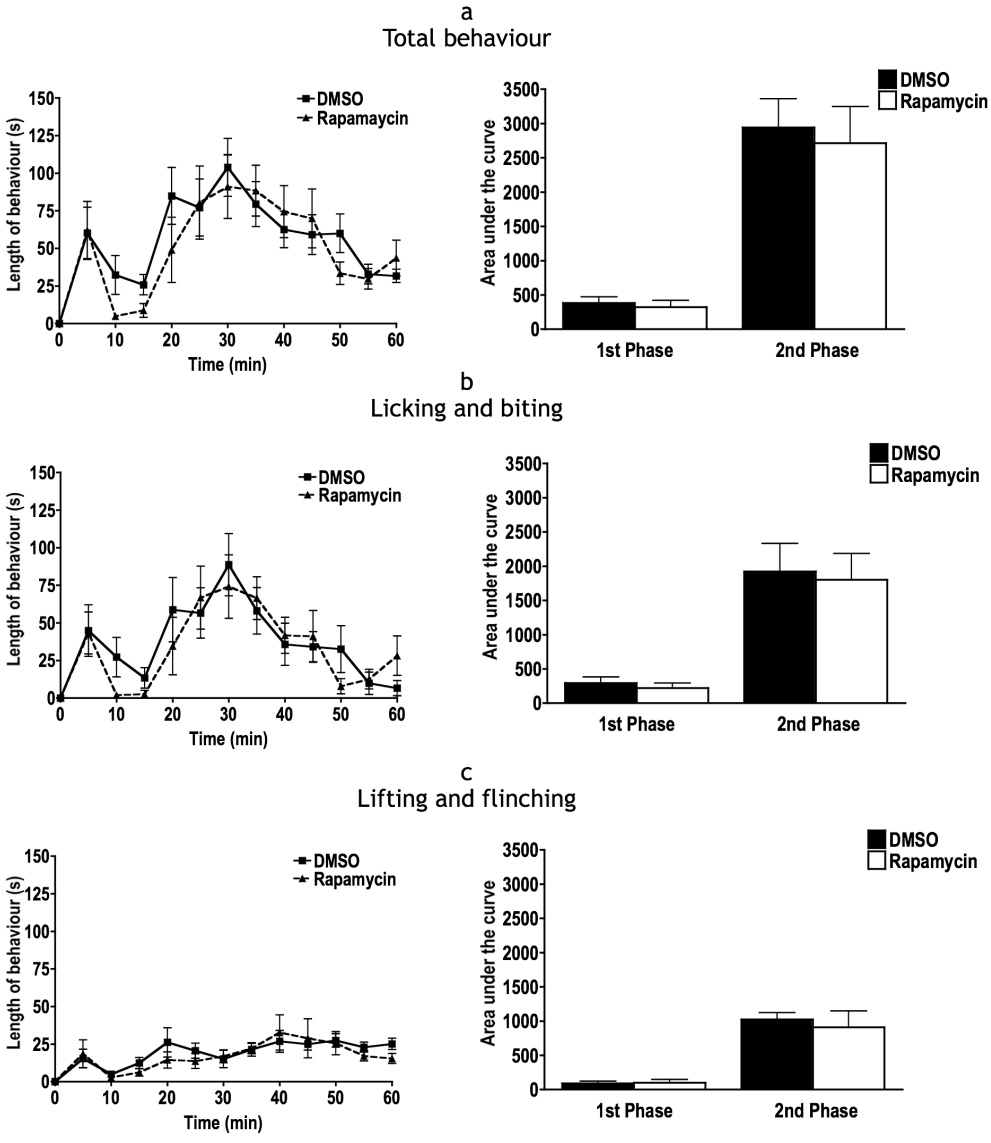


Figure 4.4 Effects of a lumbar injection of rapamycin on formalin-induced behavioural hypersensitivity when applied 5 min prior to formalin injection. (a) Total behaviour, (b) licking and biting and (c) lifting and flinching were all unaffected when pre-treated with rapamycin (n = 7) compared to DMSO (n = 7).

Although 5 min was adequate time for arousal from anaesthesia, it was likely that the anaesthesia was still present in the system of the rat thus masking the effects of rapamycin. Therefore, a longer recovery period of 20 min between rapamycin injection and formalin injection into the hind paw was allowed.

4.3.6 Rapamycin attenuates total formalin-induced behavioural hypersensitivity when administered 20 min before formalin

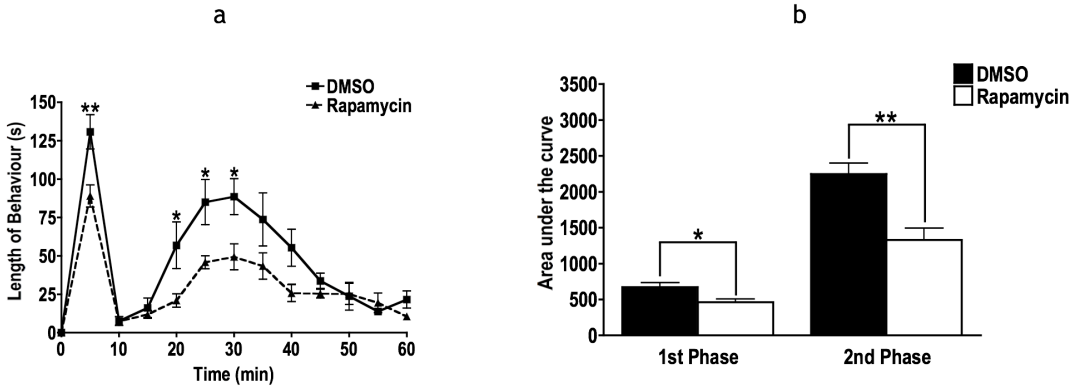


Figure 4.5 Effects of a lumbar injection of rapamycin on formalin-induced total behavioural hyperexcitability when applied 20 min prior to formalin injection. (a) When a lumbar dose of rapamycin (n = 6) was administered to rats 20 min prior to formalin injection into the hind paw, there was a significant reduction in length of total behaviour at 5, 20, 25 and 30 min when compared to DMSO (n =7) from 131 ± 11 to 89 ± 7; 57 ± 15 to 21 ± 4; 85 ± 15 to 46 ± 4 and 89 ± 12 to 49 ± 8 s respectively. AUC analysis revealed that overall, rapamycin elicited a significant reduction in the first phase from 673 ± 63 to 463 ± 42 arbitrary units. Rapamycin also elicited a significant reduction in the second phase from 2,248 ± 151 to 1,330 ± 166 arbitrary units.

4.3.7 Rapamycin attenuates licking and biting pain-like behaviour when administered 20 min before formalin

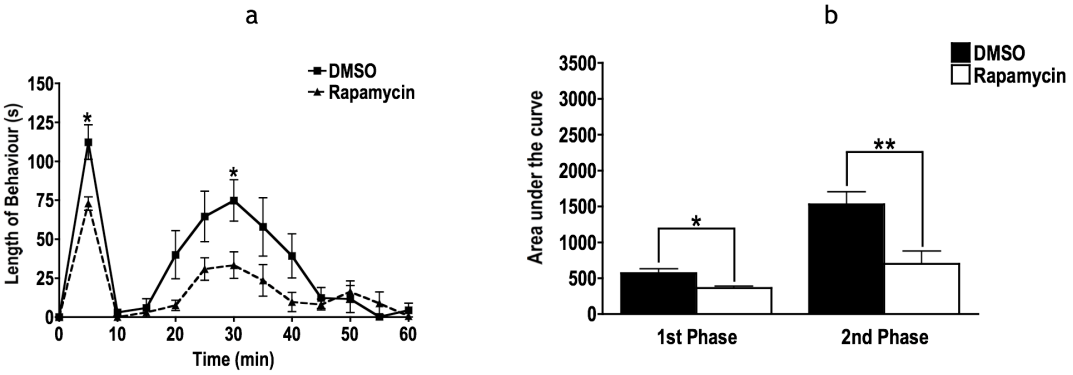


Figure 4.6 Effects of a lumbar injection of rapamycin on formalin-induced licking and biting pain-like behaviour when applied 20 min prior to formalin injection. (a) When a lumbar injection of rapamycin (n = 6) was administered to rats 20 min prior to formalin injection into the hind paw, there was a significant reduction in the length of licking and biting behaviour at 5 and 30 min when compared to DMSO (n = 7) from 112 ± 11 to 73 ± 4 and 75 ± 13 to 33 ± 9 s respectively. AUC analysis revealed that overall, rapamycin elicited a significant reduction in the first phase from 569 ± 61 to 364 ± 26 arbitrary units. Rapamycin also elicited a significant reduction in the second phase from 1,529 ± 174 to 699 ± 179 arbitrary units (*P<0.05; **P<0.01).

4.3.8 Rapamycin is ineffective against lifting and flinching pain-like behaviour when administered 20 min before formalin

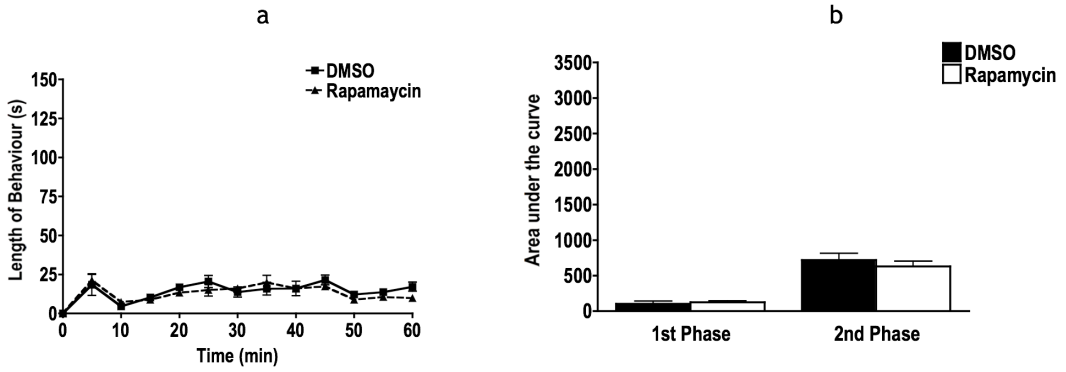


Figure 4.7 Effects of a lumbar injection of rapamycin on formalin-induced lifting and flinching pain-like behaviour when applied 20 min prior to formalin injection. (a) There were no differences at any specific time points between DMSO (n = 7) and rapamycin (n = 6) pre-treated rats. (b) Overall, there were no differences for first and second phase responses between DMSO and rapamycin pre-treated rats.

To summarise, rapamycin attenuated both neuronal hyperexcitability and behavioural hypersensitivity when compared to DMSO. In vivo electrophysiology studies revealed that rapamycin was effective if administered i.t. onto the exposed spinal cord 3 min prior to formalin injection into the hind paw and its inhibitory actions were specific for the second phase of the formalin test. These effects correlated with the results seen with the general translation inhibitor anisomycin. However, behaviourally, a single lumbar injection of rapamycin was ineffective in attenuating formalin-induced behavioural hypersensitivity when given 5 min prior to formalin injection into the hind paw, yet effective in attenuating both phases of the formalin test when given 20 min prior to the formalin injection.

4.4 Discussion

The results of these studies provide convincing evidence that rapamycin-sensitive protein translation pathways are important in the induction of formalin-induced neuronal excitability and also formalin-induced behavioural hypersensitivity and therefore may also be important in the induction of more persistent and chronic pain states.

It can be seen from the electrophysiology results that i.t. administration of rapamycin (see figure 4.2) or anisomycin (see figure 4.3) 3 min prior to formalin injection into the hind paw results in a significant attenuation in neuronal hyperexcitability in the second phase of the formalin test. A time of 3 min incubation with rapamycin was chosen due to the fact that direct evidence from studies on the importance of rapamycin-sensitive pathways in hippocampal LLTP points towards a time-restricted role for the involvement of rapamycin-sensitive pathways (Cammalleri et al., 2003). In these studies, it was found that when rapamycin (20 nM) was transiently (40 min) applied during LLTP induction of hippocampal slices, there was a dramatic decay of field excitatory postsynaptic potentials. However, a transient rapamycin application 5 min after the delivery of the LLTP tetanisation paradigm or 2 hr after completion of the LLTP induction paradigm did not alter LLTP.

Although rapamycin and anisomycin have very similar effects on the second phase of neuronal hyperexcitability, their response profiles do appear to be markedly different. Specifically, it appears that the response profiles for DMSO (25 %) in the rapamycin-formalin experiment (see figure 4.2) are lower in terms of neuronal hyperexcitability than the response profile for DMSO (10%) in the anisomycin-formalin experiment (see figure 4.3). Although DMSO was shown in chapter 3 not to have an effect on electrical and natural stimulus evoked responses compared to pre-drug controls, recent studies have highlighted the effects of DMSO on the formalin test (Colucci et al., 2008). In these studies, it was found that 100 % DMSO or DMSO:saline 1:3 (v/v) administered to mice either centrally via an intracerebroventricular (i.c.v.) injection or orally (p.o.) was able to significantly

inhibit the first and second phase via i.c.v. administration or only the second phase via p.o. administration. Thus, although DMSO may not have a significant effect on neuronal responses in naive animals, this may change in animals with an induced pain-like state. Also, there was a greater degree of second phase inhibition observed with rapamycin (see figure 4.3) compared with anisomycin (see figure 4.5). However, this may just be due to a difference in potency as well as differing DMSO percentages. Importantly, the comparison with anisomycin indicates that rapamycin does indeed act via inhibition of protein translation.

In an attempt to replicate these results behaviourally, pilot studies were carried out whereby rats were first lightly anaesthetised before receiving a single lumbar injection of rapamycin between the L5 - L6 vertebral interspace. The rats were then allowed to recover for 3 min before administering formalin to the hind paw. However, it was found that 3 min was not sufficient to allow the rats to fully regain consciousness from the anaesthesia (data not shown). Therefore, a time of 5 min was chosen, as all animals appeared to be fully conscious by this time. However, it was found that a lumbar injection of rapamycin 5 min prior to formalin injection did not replicate the results seen with in vivo electrophysiology (see figure 4.4). This meant that either the dose was insufficient to alter behavioural hypersensitivity; that the drug did not have enough time to exert its effects or that the rats had still not fully recovered from the anaesthesia. In this case, the latter was probably true. The behavioural study was repeated, but this time, the same dose of rapamycin was injected 20 min prior to formalin injection into the hind paw. On this occasion, there was a significant attenuation of the second phase of behavioural hypersensitivity, which confirmed the in vivo electrophysiology results, but there was also a significant inhibition of the first phase too (see figures 4.5 – 4.7). At first, this might appear to be due to a time related issue concerning drug action behaviourally. However, closer observation of the two response profiles presents a different view. When formalin was injected 5 min after a lumbar injection of rapamycin, the first phase was markedly lower than when formalin was injected 20 min after a lumbar injection of rapamycin. This is interesting because it is the first phase, which is attributed to acute afferent activity in response to direct activation of peripheral nociceptors that leads to onset of the second phase

which involves longer-lasting changes in plasticity of spinal neurones as well as descending controls from higher brain centres (Suzuki et al., 2004a). The fact that there was such a contrast between the first phase responses depending upon the length of recovery time prior to formalin injection indicates that although the rats may have seemed fully conscious, there may have been residual systemic anaesthetic isofluorane still present in high enough levels that were sufficient to dampen down the response to formalin as well as mask the inhibitory effects of rapamycin.

It is interesting that rapamycin-sensitive pathways are important in both phases of the formalin test as this provides important information regarding the role of rapamycin-sensitive pathways in both peripheral and central aspects of pain processing. There is also strong electrophysiological evidence showing that descending serotonergic controls from higher brain centres are also enhanced during formalin-induced inflammation, acting via excitatory 5-HT3Rs in the dorsal horn to maintain or even enhance the response of spinal neurones to formalin-induced inflammation since the selective 5-HT3R antagonist ondansetron and SP-SAP treatment (both i.t.) prior to formalin injection into the hind paw both attenuate the neuronal hyperexcitability associated with formalin-induced inflammation (Green et al., 2000; Suzuki et al., 2002). In support of a spinal site of action for rapamycin-sensitive pathways and their activation by descending serotonergic pathways acting at spinal 5-HT3Rs, is a study whereby an i.t. dose of the selective 5-HT3R antagonist ondansetron was shown to attenuate formalin-induced behavioural hypersensitivity as well as the upstream regulator of mTOR, ERK in the spinal cord (Svensson et al., 2006).

Also of interest is that fact that behaviourally, rapamycin is more effective in reducing licking and biting as opposed to lifting and flinching. According to optimal scoring strategies (Watson et al., 1997), licking and biting has a higher categorical weight than lifting and flinching. One can imagine that lifting and flinching behaviour comprises a significant proportion of reflex behaviour whereas licking and biting may require much more conscious processing by the rat with the aim of alleviating the behavioural hypersensitivity. Importantly, the behavioural data

confirm that rapamycin-sensitive pathways are important in formalin-induced behavioural hypersensitivity.

5 Rapamycin-sensitive pathways and pathophysiological conditions: spinal nerve ligation-induced neuropathy

5.1 Introduction

In 1994, neuropathic pain was defined as "pain initiated or caused by a primary lesion or dysfunction of the nervous system" by the International Association for the Study of Pain (IASP) taxonomy committee (Merskey and Boduk, 1994). However, a report by Treede et al. has highlighted the need for this definition to be revised and a new definition be created using the following wording "pain arising as a direct consequence of a lesion or disease affecting the somatosensory system". With this definition, the term 'dysfunction', which is not well defined, is replaced with the term 'disease', which refers to specific identifiable processes such as inflammation, autoimmune conditions and channelopathies. The term nervous system is replaced with the term somatosensory system because diseases and lesions in other parts of the nervous system may cause other types of pain such as that associated with spasticity and rigidity mediated by activation of muscle nociceptive afferents that may be wrongly confused with neuropathic pain (Treede et al., 2008).

5.1.1 The clinical situation

The more common peripheral and central neuropathic conditions associated with neuropathic pain are shown in table 5.1. It is important to note that these conditions will not always result in pain and the reasons behind this are not clear. Neuropathic pain is thought to affect a small but significant percentage of the UK population and although a precise number is not defined, there have been numerous studies investigating incidence rates of particular neuropathic conditions and their treatment. One such study carried out by Hall et al. determined the incidence of four readily diagnosed pain syndromes: post-herpetic neuralgia (PHN), trigeminal neuralgia, phantom limb pain (PLP) and painful diabetic neuropathy (PDN) as managed in general practice (Hall et al., 2006). Over a 10 yr observational period involving 39,731 patients for whom, a record of one or more of the four pain syndromes had been identified, there were 12,386 incident cases of PHN

(31 %), 8,268 cases of trigeminal neuralgia (21 %), 451 cases of PLP (5 %) and 4719 (12 %) cases of PDN. Hall et al. also studied treatment regimens and found that many different drugs were prescribed as initial treatments, but the five most commonly used were the same across all conditions: analgesics, tricyclic antidepressants (TCAs), anticonvulsants, paracetamol and codeine. Data regarding the number of changes from initial to stable therapy suggests that initial drug choice is not perfect and that those patients whose initial drug treatment was with conventional analgesics such as opioids and paracetamol were more likely to change treatment than those whose initial drug was either an antidepressant such as amytriptilline or an anticonvulsant such as carbemazepine (Hall et al., 2006).

In 2006, the European Federation of Neurological sciences (EFNS) panel on neuropathic pain produced a set of guidelines based on the evaluation of existing evidence about the pharmacological treatment of neuropathic pain. They found strong evidence for the efficacy of TCAs, the anticonvulsants GBP and pregabalin and opioids for PHN and peripheral polyneuropathies (PPN) caused mainly by diabetes. These drugs are recommended for first line treatment. Topical lidocaine, the serotonin-noradrenaline reuptake inhibitors (SNRIs) such as venlafaxine and duloxetine were found to have less established efficacy and are therefore recommended for second line treatment. Opioids are suggested for second to third line treatment in chronic non-cancer pain (Attal et al., 2006). Despite these guidelines and the large number of trials, neuropathic pain treatment remains unsatisfactory for the many patients that endure a trial and error process before obtaining some kind of satisfactory regimen that may often combine more that one drug treatment.

Peripheral trauma	Central trauma
Traumatic (including iatrogenic) nerve injury	Stroke (infarct or haemorrhage)
Ischaemic neuropathy	Multiple sclerosis
Nerve compression/entrapment	Spinal cord injury
Polyneuropathy (hereditary, metabolic, toxic, inflammatory, infectious, paraneoplastic, nutritional, or in amyloids or vasculitis)	Syringomyelia/syringobulbia
Plexus injury	
Root compression	
Stump and phantom pain after amputation	
PHN	
Cancer-related neuropathy (i.e. due to neural invasion of the tumour, surgical nerve damage, radiation-induced nerve damage, or chemotherapy-induced neuropathy)	
Scar pain	

Table 5.1 Some common conditions in which neuropathic/neurogenic pain may appear (adapted from Hansson, 2008).

5.1.2 Animal models of neuropathic pain

In light of the fact that neuropathic pain treatment for many patients is unsatisfactory, basic studies using animal models have been used to provide clues into the mechanism of action of many drugs as well as improve current drug treatment regimes. So far, the most intensively studied animal models for neuropathic pain are those involving a mechanical trauma to peripheral nerves (Zimmermann, 2001). The past 20 years have provided a multitude of rodent neuropathic pain models that correspond with the various existing clinical conditions. In terms of peripheral nerve injury, three commonly used mononeuropathy models that simulate human neuropathic pain syndromes are the chronic constriction injury (CCI) model, the partial sciatic nerve ligation (PSL) model and the spinal nerve ligation (SNL) model.

The rat CCI model was created by Bennett and Xie in 1988 and involves loosely tying the left or right sciatic nerve with four chromic gut ligatures at the mid-thigh level (Bennett and Xie, 1988). Then came the rat PSL model by Seltzer et al. in 1990. This model involves ligating the sciatic nerve at the high thigh level, ensuring that a third to half the thickness of the sciatic nerve is ligated (Seltzer et al., 1990). In 1992, Kim and Chung reported on their rat model of neuropathic pain. In this model, both the L5 and L6 spinal nerves on one side of the rat are tightly ligated at a site distal to the DRG (Kim and Chung, 1992). Kim and Chung also described a commonly used modification of this model that involves tight ligation of the L5 spinal nerve only, a model which also exhibits long-lasting hypersensitivity (Kim and Chung, 1992).

All three models of neuropathic pain show behavioural signs of both mechanical and cold hypersensitivity as well as spontaneous pain, although in terms of the magnitude of responses, there is some variation between the different models. In a study comparing these particular models, it was found that responses to mechanical stimuli were the highest in the SNL model and lowest in the CCI model. The responses to cold stimuli were similar in magnitude in all three models. However, there was much more variability between animals in the CCI model (Kim et al., 1997). Taking this information into consideration, the studies presented

here will involve the SNL model (ligation of L5 and L6 spinal nerves) due to the large and stable magnitude of stimulus evoked behavioural hypersensitivity. In addition to behavioural hypersensitivity, electrophysiological studies of neuronal activity have shown that SNL alters neuronal plasticity and particularly enhances ongoing neuronal activity such that pharmacological agents that inhibit neuronal activity- some of which are used clinically, are more efficacious in this pain-like state compared to sham or naive animals (Suzuki et al., 1999; Suzuki et al., 2000; Suzuki et al., 2001; Suzuki et al., 2004b; Suzuki et al., 2005). Furthermore, there is little variability between individual rats of this model (Kim and Chung, 1992) which is presumably due to the fact that single ligatures are being placed on a single spinal nerve as opposed to a number of ligatures covering a comparatively larger area of sciatic nerve (as is the case with CCI), or a ligature of a variable section (third to half) of the sciatic nerve (as is the case with PSL).

5.1.3 Animal models of neuropathic pain and upstream modulators of mTOR

Much like formalin-induced inflammation, there is also little information on the importance of rapamycin-sensitive pathways in neuropathy, although more is known regarding animal models of neuropathic pain and upstream regulators of mTOR. ERK activation has been shown to be important in engaging or synergising with rapamycin-sensitive pathways (Gelinas et al., 2007; Kelly et al., 2007; Tsokas et al., 2007). As well as being important in inflammatory pain-like conditions, ERK activation has also been shown to be important in mediating pain-like hypersensitivity induced by nerve injury (Zhuang et al., 2005). In this particular study, Zhuang et al. found that an L5 spinal nerve ligation of rats induced activation of ERK via phosphorylation in the superficial neurones of the dorsal horn of the spinal cord as well as astrocytes and microglia. Furthermore, i.t. administration of the inhibitor of ERK kinase (which is upstream to ERK)- PD98059 to these rats caused significant reductions in mechanically evoked hypersensitivity. PI3K is also upstream of mTOR and its role in activating rapamycin-sensitive pathways has been well documented (Schratt et al., 2004; Takei et al., 2004; Horwood et al., 2006; Chenal and Pellerin, 2007; Kelly et al., 2007; Tsokas et al., 2007). Little is known about the role of PI3K in nerve injury-induced behavioural

hypersensitivity although a recent study addressed this by exploring the role of PI3K signalling in mechanical and thermal hypersensitivity after ligation of the L5 spinal nerve. By measuring downstream Akt phosphorylation levels, it was found that there was an increase in PI3K activity in the dorsal horn and DRG ipsilateral to the injury. To further substantiate this, the PI3K inhibitors wortmanin or LY294002 were shown to attenuate mechanical and thermal behavioural hypersensitivity (Xu et al., 2007).

At the receptor level, NMDA receptors are implicated in activating rapamycin-sensitive pathways (Gong et al., 2006; Gonzalez-Mejia et al., 2006) and have also been studied for their role in nerve-injury induced behavioural hypersensitivity. In a study examining the efficacy of specific NMDA receptor antagonists on the L5 and L6 SNL model, it was found that the antagonists memantine, D-AP5, dextrophan, dextromethorphan, MK801 and ketamine were effective (to varying degrees) in attenuating SNL-induced mechanical behavioural hypersensitivity (Chaplan et al., 1997). In accordance with these results, electrophysiological data studies on the same models have demonstrated that systemic administration of the NMDA antagonists memantine, ketamine and MK801 are effective (again to varying degrees) in reducing post-discharge, wind up as well as thermally and mechanically evoked responses from SNL rats compared to sham controls (Suzuki et al., 2001).

Much like formalin-induced inflammation, the metabotropic glutamate receptors mGluR1 and mGluR5 have also been shown to be important in SNL-induced behavioural hypersensitivity (Dogrul et al., 2000; Varty et al., 2005) and they are known to be important in engaging rapamycin-sensitive pathways (Page et al., 2006; Price et al., 2007). In 2000, Dogrul et al. showed that the selective mGlur5 antagonist SIB-1757 was effective in fully reversing SNL-induced thermal behavioural hypersensitivity when administered via an i.t. lumbar spinal injection or locally to the hypersensitive hind paw (Dogrul et al., 2000). Later in 2005, it was shown that the mGluR5 antagonists MPEP, MTEP and the mGluR1 antagonist LY456236 were all effective in attenuating SNL-induced mechanical hypersensitivity when administered i.p. (Varty et al., 2005).

At the transmitter level, BDNF has been shown to be important in engaging rapamycin-sensitive pathways (Takei et al., 2001; Tang et al., 2002; Schratt et al., 2004; Takei et al., 2004) as well as modulating the effects of nerve injury-induced behavioural hypersensitivity. In a study by Yajima et al., it was found that PSL-induced behavioural thermal hypersensitivity and mechanical hypersensitivity could be suppressed by repeated i.t. injections of the BDNF sequestering protein TrkB/Fc. In addition, heterozygous BDNF KO mice presented with a significantly lower degree of PSL-induced thermal and mechanical hypersensitivity compared to WT mice (Yajima et al., 2005).

5.1.4 Direct nerve injury and rapamycin-sensitive pathways

It is widely accepted that nerve damage experienced by neuropathic pain patients leads to various changes in the excitability of these nerves which contributes to the symptoms commonly associated with neuropathic pain i.e. allodynia, hyperalgesia dysasthesia and even loss of sensation. Although the importance of rapamycin-sensitive pathways in neuropathy has not been extensively studied, much can be learned from the first demonstration of the importance of rapamycin-sensitive pathways in nerve-injury induced hyperexcitability (Weragoda et al., 2004). In this study involving easily identifiable neurons from *Aplysia californica* (Californian sea slug), intracellular recordings from the somata of sensory and secretomotor neurones were found to exhibit stimulus evoked hyperexcitability (LTH) lasting at least 24 hr when they were either crushed or briefly exposed (2 min) to elevated extracellular [K^+]. This hyperexcitability was prevented when the nerve segments were incubated for 3 hr prior to the treatment with rapamycin. Subsequently, in 2005, it was also reported that this crush-induced LTH could be prevented by incubating the nerve segments with the 5-HTR antagonist methiothepin, providing evidence for the mediation of this response by 5-HT (Weragoda and Walters, 2007), as well as an interesting correlation with animal models of pain where descending serotonergic influences have been to shown to play a significant role in maintaining persistent and chronic pain-like states (Suzuki et al., 2004a).

More recently, rapamycin-sensitive pathways have been shown to be important in the rat spared nerve injury (SNI) model, whereby mechanical hypersensitivity

occurs as a result of ligation and then removal of the peroneal and tibial nerve stumps that branch off the sciatic nerve (Jimenez-Diaz et al., 2008). In this study, it was found that this injury resulted in an enhanced response to pinprick stimulation in the hind paw as determined by the amount of time holding the paw. When rapamycin was injected into the intraplantar region of the hind paw, there was a significant decrease in time holding the paw 4 to 24 hr post-injection. To date, there have been no studies investigating the importance of rapamycin-sensitive pathways at the spinal level in rodent models of neuropathy and this was the aim here. The studies presented in this chapter utilise in vivo electrophysiology and behavioural studies techniques to investigate this further using the rapamycin ester CCI-779.

5.2 Methods

5.2.1 SNL surgery

Surgery was performed as described in 2.4.1.

5.2.2 Behavioural studies: confirmation of pain-like behaviour and assessment of CCI-779 efficacy

See 2.4.2. When assessing the efficacy of CCI-779, 12.88 μg in 20 μl saline (250 μM) was injected into the lumbar region of lightly anaesthetised rats, which were then allowed to recover (20 min) before testing began. This dose was chosen as it had been shown to attenuate capsaicin- and nerve injury-induced behavioural hypersensitivity when injected locally into the hind paw (Jimenez-Diaz et al., 2008). The total testing period was 2 hr.

5.2.3 In vivo electrophysiology setup

See 2.2. After obtaining a stable baseline, 12.88 ng of CCI-779 in 50 μl of saline was applied to the exposed spinal cord and electrically and naturally evoked stimuli were obtained every 20 min for 2 hr.

5.2.4 Statistical analysis

To confirm pain-like behaviour, comparisons were made between sham and SNL difference scores using the Kruskal-Wallis test with Bonferroni's post-tests. When determining the maximal effects of CCI-779 compared to saline within the first hr of administration to SNL animals, AUC response profiles were constructed for all difference scores within the first hr and CCI-779 AUC data was compared to saline AUC data using Mann-Whitney tests. Electrophysiological raw data are presented as mean $\pm$ SEM response. Student's unpaired t-tests were used to compare sham and SNL pre-drug baseline control neuronal responses with each other, except for graded thermally and mechanically evoked responses where two way ANOVA with Bonferroni post-tests were used. For time course data, responses at a given time point were compared to t = 0, when CCI-779 was added to the cord. One way ANOVA with repeated measures and Dunnett's post-test comparisons to control (at

0 min) were used to determine significance between groups for electrical stimuli as well as brush and pin. Two way ANOVA with repeated measures and Bonferroni's post-test comparisons to control (at 0 min) were used to determine significance between groups for graded natural stimuli i.e. graded mechanical and thermal stimuli. In addition, maximal stimulus evoked responses (positive or negative) to drug administration were analysed. For drug-induced maximal response changes to electrical, brush and pinprick stimuli, one way ANOVA with Bonferroni multiple comparisons post-tests were used. For maximal graded mechanical and thermal stimuli evoked responses, two way ANOVA with Bonferroni multiple comparisons post-tests were used (*$P<0.05$; **$P<0.01$ ***$P<0.001$).

5.3 Results

5.3.1 SNL induces persistent pain-like behaviour post-surgery

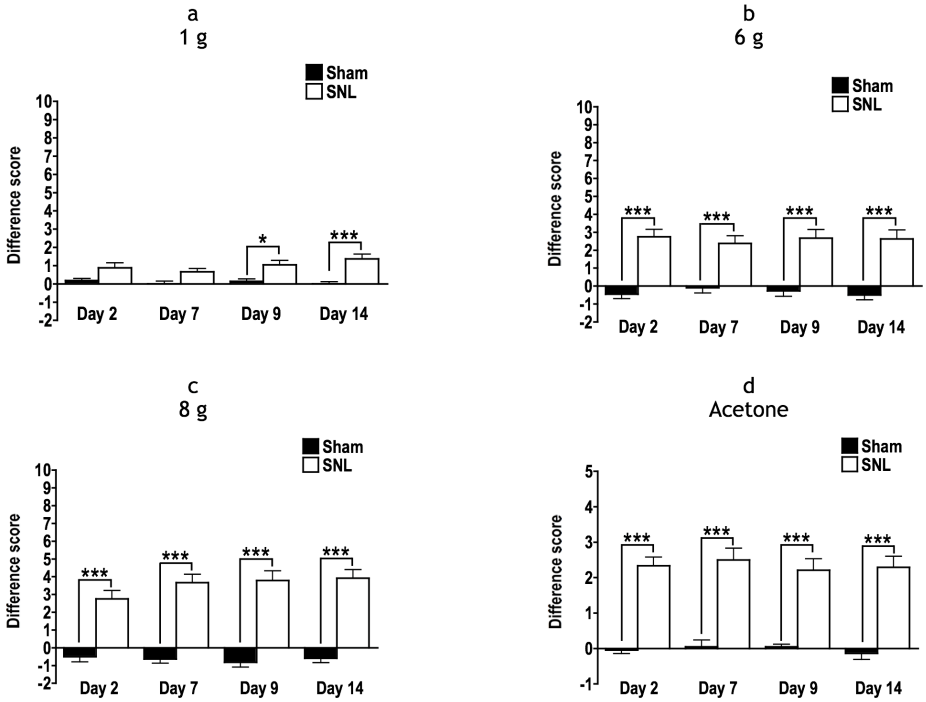


Figure 5.1 Effects of SNL on stimulus evoked behavioural withdrawal responses. (a) When a 1 g von Frey stimulus was applied 10 times to each hind paw, there was an increase in behavioural hypersensitivity in SNL rats (n = 22) compared to sham rats (n = 24) as demonstrated by the increase in difference score. This increase was significant at post-surgery days 9 and 14, where the difference score increased from 0.19 ± 0.14 and 0 ± 0.12 to 1.04 ± 0.24 and 1.38 ± 0.26 respectively. (b) When 6 g was applied, there was a significant increase in difference score at 2, 7, 9 and 14 days from –0.45 ± 0.25 to 2.75 ± 0.41; -0.09 ± 0.29 to 2.38 ± 0.43; -0.27 ± 0.30 to 2.67 ± 0.48 and –0.50 ± 0.27 to 2.63 ± 0.5 respectively. (c) When 8 g was applied, there was a significant increase in difference score at 2, 7, 9 and 14 days from –0.50 + 0.30 to 2.75 ± 0.47; -0.64 ± 0.24 to 3.67 ± 0.47; -0.82 ± 0.27 to 3.79 ± 0.55 and –0.59 ± 0.25 to 3.92 ± 0.48 respectively. (d) When acetone was applied, there was a significant increase in difference score at 2, 7, 9 and 14 days from –0.05 ± 0.10 to 2.33 ± 0.25; 0.05 ± 0.19 to 2.50 ± 0.33; 0.05 ± 0.01 to 2.21 ± 0.32 and –0.14 ± 0.17 to 2.29 ± 0.31 respectively. Difference score (see 2.4.2.) = number of paw withdrawals from injured side minus noumber of paw withdrawals from uninjured side (*P<0.05, **P<0.01, ***P<0.001).

5.3.2 CCI-779 is not effective against electrically evoked neuronal responses from SNL rats

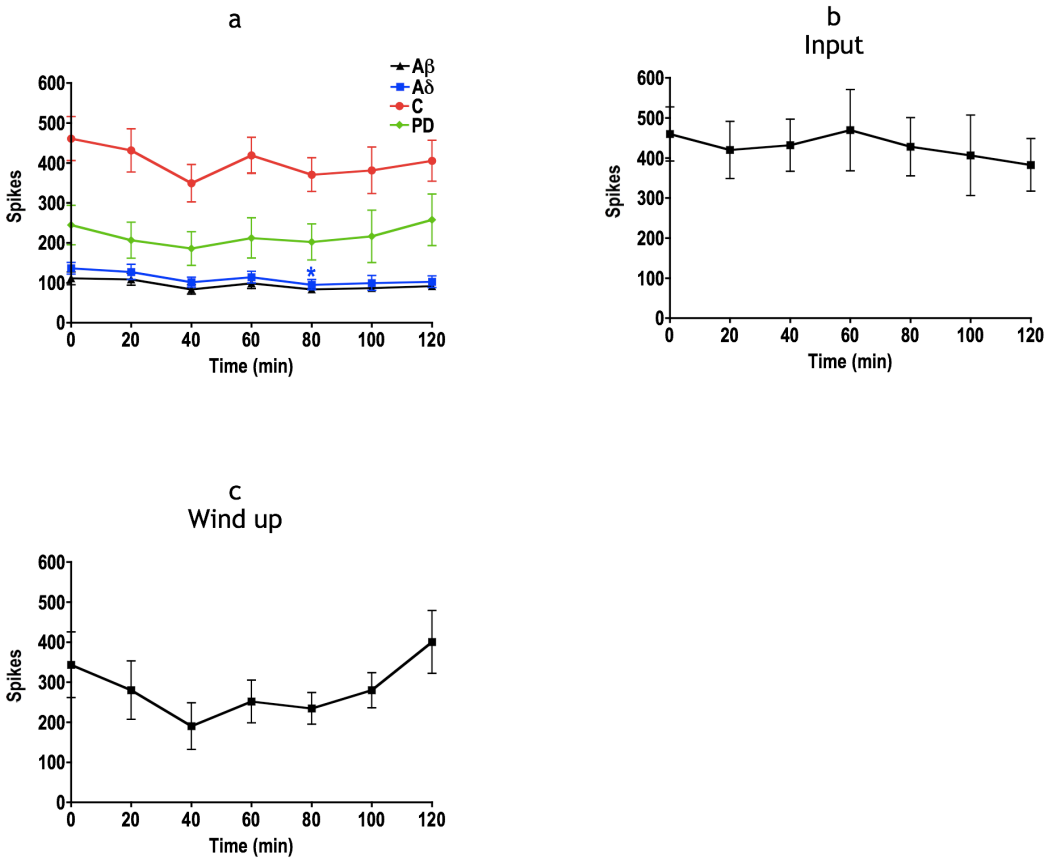


Figure 5.2 Effects of 250 nM CCI-779 on electrically evoked neuronal responses from SNL rats over 2 hr. (a) Only Aδ-fibre activity was significantly inhibited at 80 min from 136 ± 15 (at 0 min) to 95 ± 12 spikes. There were trends for inhibition within the first hr for: (a) Aβ-, Aδ-, C-fibre-mediated activity and post discharge (PD); (b) input and (c) wind up, particularly at 40 min. For all data sets, spikes = number of spikes after a train of 16 pulses; n = 12, except wind up, where n = 11 (*P<0.05).

5.3.3 CCI-779 is not effective on electrically evoked neuronal responses from sham rats

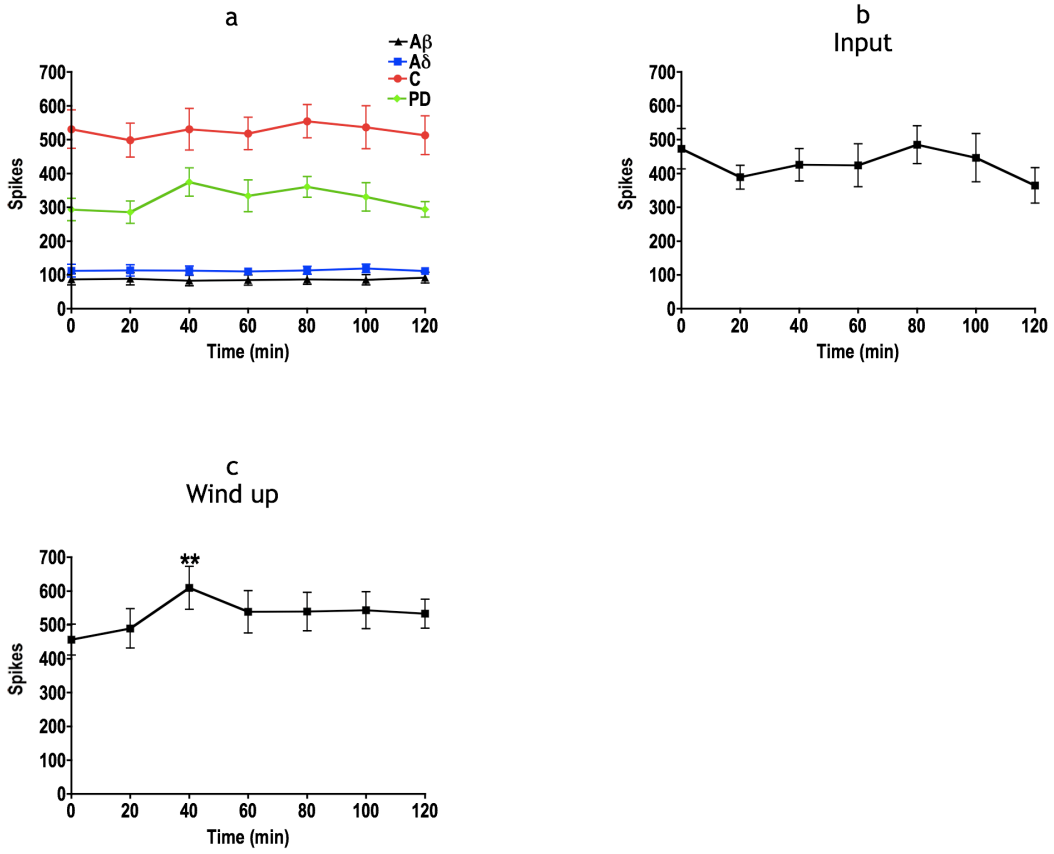


Figure 5.3 Effects of 250 nM CCI-779 on electrically evoked neuronal responses from sham rats over 2 hr. Much like SNL rats, electrically evoked responses were on the whole unchanged for (a) Aβ-, Aδ-, C-fibre-mediated activity, post discharge (PD) and (b) input. However, there were trends for facilitation rather than inhibition seen with SNL rats, particularly at 40 min. This was more apparent with (c) windup where there was an increase at 40 min from 457 $\pm$ 46 (at 0 min) to 610 $\pm$ 64 spikes. For all data sets, spikes = number of spikes after a train of 16 pulses; n = 10 (**P<0.01).

5.3.4 CCI-779 has small effects on brush but not pinprick evoked neuronal responses from SNL rats

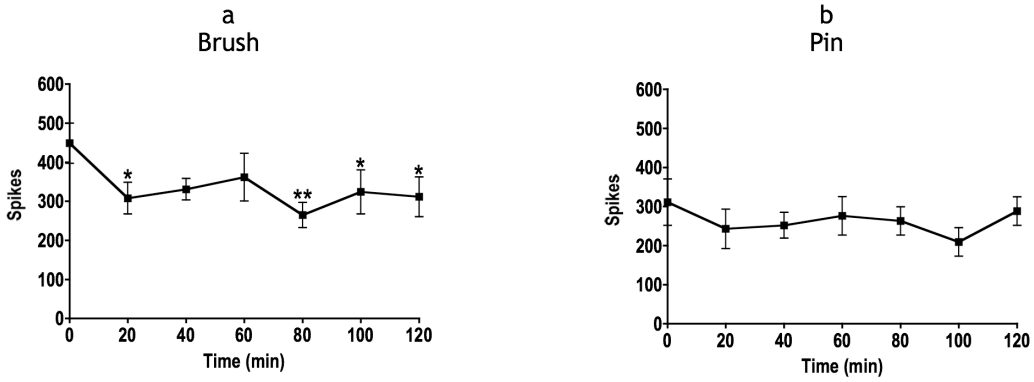


Figure 5.4 Effects of 250 nM CCI-779 on brush and pinprick evoked neuronal responses from SNL rats over 2 hr. (a) When CCI-779 was administered to the spinal cord, there was a reduction in brush evoked activity from 449 $\pm$ 51 (at t = 0) to 308 $\pm$ 41; 265 $\pm$ 32; 324 $\pm$ 56 and 312 $\pm$ 51 at 20, 80, 100 and 120 min respectively. (b) There was no effect on pinprick evoked stimuli. For both data sets, spikes = number of spiked during a 10 s stimulus; n = 12 (*P<0.05; **P<0.01).

5.3.5 Inhibitory effects of CCI-779 on brush evoked neuronal responses are absent from sham rats

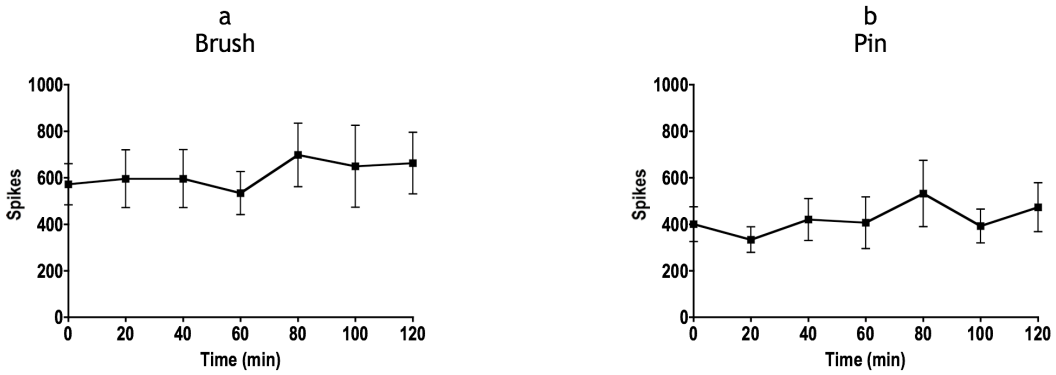


Figure 5.5 Effects of 250 nM CCI-779 on brush and pinprick evoked neuronal responses from sham rats over 2 hr. When CCI-779 was administered to the spinal cord, there were no effects on (a) brush evoked responses or (b) pinprick evoked responses. For both data sets, spikes = number of spikes during a 10 s stimulus; n = 10.

5.3.6 CCI-779 has selective effects on graded mechanically and thermally evoked neuronal responses from SNL rats

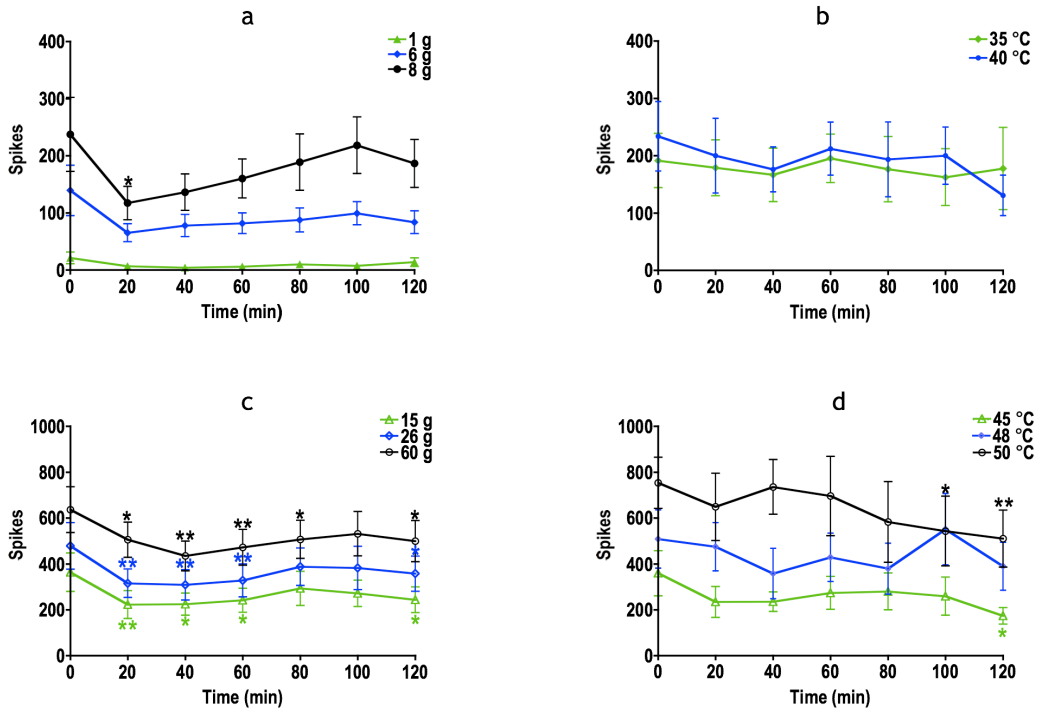


Figure 5.6 Effects of CCI-779 on graded mechanically and thermally evoked neuronal responses from SNL rats over 2 hr. (a) At 20 min, 250 nM CCI-779 specifically inhibited responses to 8 g from 237 ± 65 (at t = 0) to 117 ± 29 spikes. (b) CCI-779 had no effect on innocuous thermally evoked stimuli. (c) At 20 min, CCI-779 inhibited responses to 15, 26 and 60 g from 364 ± 84, 479 ± 102 and 637 ± 100 spikes (at 0 min) to 223 ± 61, 316 ± 62 and 506 ± 77 spikes respectively. At 40 min, CCI-779 inhibited responses to 15, 26 and 60 g from 364 ± 84, 479 ± 102 and 637 ± 100 spikes (at 0 min) to 225 ± 48, 309 ± 66 and 435 ± 65 spikes respectively. At 60 min, CCI-779 inhibited responses to 15, 26 and 60 g from 364 ± 84, 479 ± 102, 637 ± 100 spikes (at 0 min) to 241 ± 52, 328 ± 72 and 472 ± 78 spikes respectively. At 80 min, CCI-779 inhibited responses to 60 g from 364 ± 84 spikes (at 0 min) to 294 ± 68 spikes. At 120 min, CCI-779 inhibited responses to 15, 26 and 60 g from 364 ± 84, 479 ± 102 and 637 ± 100 spikes (at 0 min) to 244 ± 51, 358 ± 70 and 499 ± 82 spikes respectively. (d) At 100 min, CCI-779 inhibited responses to 50 °C from 754 ± 112 spikes (at 0 min) to 543 ± 132 spikes. At 120 min, CCI-779 inhibited responses to 45 and 50 °C from 359 ± 99 and 754 ± 112 spikes (at 0 min) to 174 ± 27 and 510 ± 91 spikes respectively. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 12 (*P<0.05; **P<0.01).

5.3.7 CCI-779 is less effective in attenuating graded mechanically and thermally evoked responses from sham rats

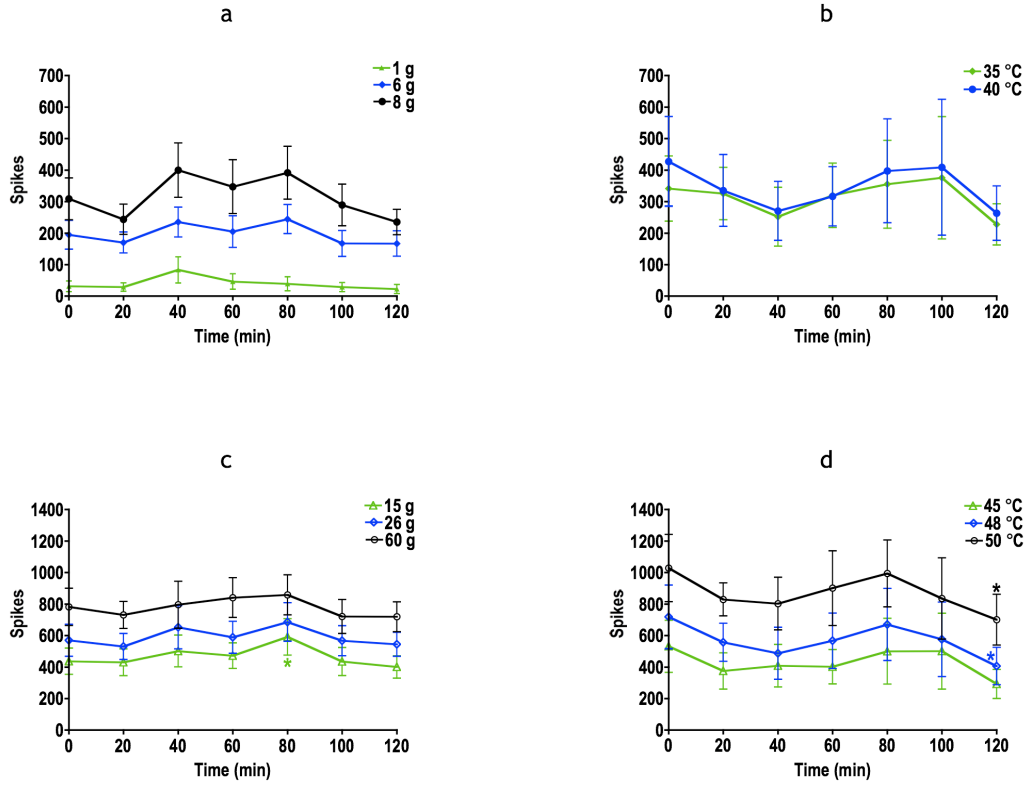


Figure 5.7 Effects of CCI-779 on graded mechanically and thermally evoked neuronal responses from sham rats over 2 hr. Although there were trends for inhibition within the first hr of testing, (a) 250 nM CCI-779 had no effect on responses to innocuous mechanically evoked responses and (b) innocuous thermally evoked responses. (c) Although CCI-779 did not inhibit responses to noxious mechanically evoked responses, there was a facilitation at 80 min to a stimulus of 15 g from 437 $\pm$ 83 spikes (at 0 min) to 591 $\pm$ 116 spikes. (d) At 120 min, CCI-779 inhibited responses to 48 and 50 °C and from 718 $\pm$ 203 and 1028 $\pm$ 214 spikes (at 0 min) to 406 $\pm$ 105 and 700 $\pm$ 142 spikes respectively. For all data sets, spikes = number of spikes during a 10 s stimulus; n = 10 (*P<0.05).

The electrophysiological results so far reveal that unlike naive rats (see chapter 3) and sham rats, there is a greater degree of inhibition of evoked responses taking place within the first hr in SNL rats and where these changes are not significant, there is certainly a strong trend for inhibition. It was therefore decided to look at the maximal changes (positive or negative) occurring for all responses evoked within the first hr of testing. The following electrophysiological data compares 3 groups: pre-drug baseline controls comprising pooled mean sham and SNL baseline responses; sham and SNL responses to CCI-779 within the first hr post-administration. Pre-drug control means were pooled into one group for sham and SNL pre-drug baseline responses called 'control' as it was found that pre-drug responses between the two groups were not significantly different.

5.3.8 Sham and SNL pre-drug baseline control neuronal responses were not significantly different

	Sham (n = 10)	SNL (n = 12)
Aβ-fibre spikes	87 ± 16	111 ± 16
Aδ-fibre spikes	112 ± 19	136 ± 15
C-fibre spikes	461 ± 55	531 ± 57
Post-discharge spikes	293 ± 33	245 ± 49
Input spikes	480 ± 64	464 ± 64
Wind up spikes	467 ± 46	344 ± 82
Brush spikes	572 ± 89	445 ± 52
Pin spikes	400 ± 75	311 ± 59
1 g spikes	31 ± 17	21 ± 10
6 g spikes	195 ± 46	125 ± 44
8 g spikes	300 ± 60	237 ± 65
15 g spikes	437 ± 83	364 ± 84
26 g spikes	570 ± 101	479 ± 102
60 g spikes	782 ± 118	637 ± 100
35 °C spikes	342 ± 104	192 ± 47
40 °C spikes	427 ± 143	234 ± 61
45 °C spikes	444 ± 167	359 ± 99
48 °C spikes	610 ± 204	509 ± 278
50 °C spikes	975 ± 208	754 ± 118

Table 5.2 Pre-drug control responses of WDR neurones from sham and SNL rats. For electrically evoked responses, a train of 16 pulses at three times C-fibre threshold (0.5 Hz, 2 ms pulse width) was applied to the corresponding hind paw. For mechanically evoked responses, von Frey filaments of ascending force were applied to the corresponding receptive field for 10 s. For thermally evoked responses, a small water jet was applied to the corresponding receptive field for 10 s. All data are expressed as raw mean values ± SEM. There were no significant differences between WDR neurone responses from sham or SNL rats prior to CCI-779 administration.

5.3.9 CCI-779 inhibits electrically evoked neuronal responses from SNL rats

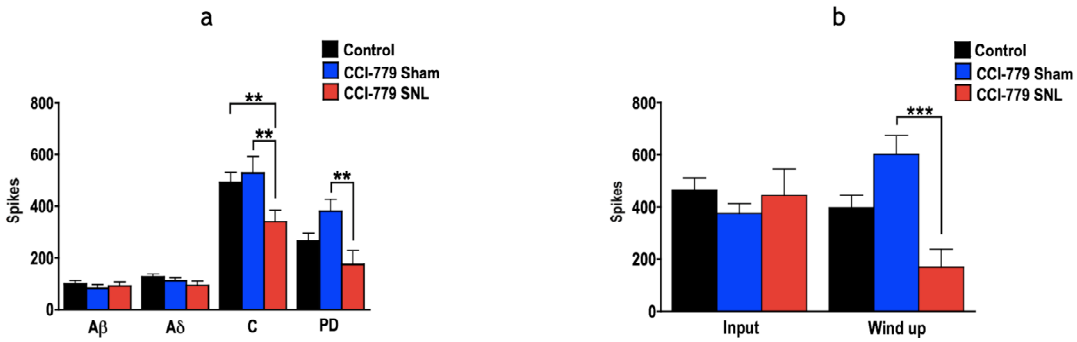


Figure 5.8 Effects of CCI-779 on electrically evoked neuronal responses from SNL and sham rats over 1 hr. (a) 250 nM CCI-779 inhibited C-fibre mediated transmission onto WDR neurones from 493 ± 39 (pre-drug controls) and 530 ± 64 (sham rats) to 340 ± 46 spikes in SNL rats. CCI-779 also inhibited post discharge of WDR neurones from 382 ± 44 (sham rats) to 176 ± 52 spikes in SNL rats. (b) CCI-779 inhibited wind up of WDR neurones from 601 ± 74 spikes (sham rats) to 172 ± 65 spikes (SNL rats). For all data sets, spikes = number of spikes after a train of 16 pulses; pre-drug sham and SNL controls n = 22; sham n = 10 and SNL n = 12 (*P<0.05; **P<0.01; ***P<0.001).

5.3.10 CCI-779 has no effects on brush and pinprick evoked neuronal responses from SNL rats

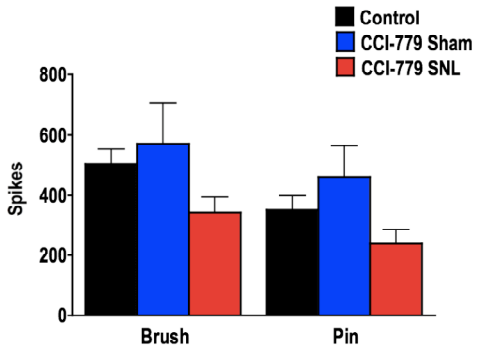


Figure 5.9 Effects of CCI-779 on brush and pinprick evoked neuronal responses from SNL and sham rats over 1 hr. Although there were trends for inhibition within the first hr in SNL rats compared with sham rats and pre-drug controls, brush evoked responses were unaltered by 250 nM CCI-779, as were pinprick evoked responses. For both data sets, spikes = number of spikes during a 10 s stimulus; pre-drug sham and SNL controls n = 22; sham n = 10 and SNL n = 12.

5.3.11 CCI-779 selectively inhibits graded mechanically evoked neuronal responses from SNL rats

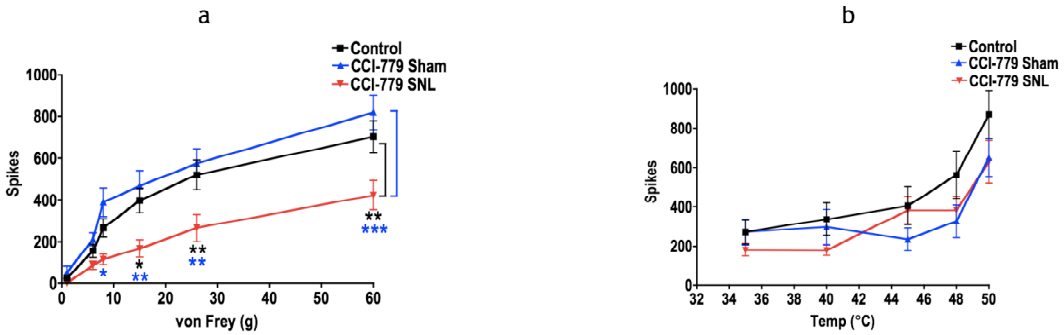


Figure 5.10 Effects of CCI-779 on graded mechanically and thermally evoked neuronal responses from SNL and sham rats over 1 hr. (a) When compared to pre-drug control responses, 250 nM CCI-779 was effective in attenuating responses to 15, 26 and 60 g from 397 ± 59, 520 ± 71 and 703 ± 76 spikes to 168 ± 41, 265 ± 65 and 422 ± 70 spikes respectively. This was also true for sham responses where, CCI-779 attenuated responses to 8 g from 388 ± 68 spikes to 116 ± 25 spikes in addition to significant inhibition of SNL responses to 15, 26 and 60 g from 467 ± 73, 575 ± 71 and 819 ± 83 spikes to 115 ± 25, 265 ± 65 and 422 ± 70 spikes respectively. (b) CCI-779 was ineffective against thermally evoked responses in SNL or sham rats compared to pre-drug control responses. For all data sets, spikes = number of spikes during a 10 s stimulus; pre-drug sham and SNL controls n = 22; sham n = 10 and SNL n = 12.

The efficacy of CCI-779 was also tested behaviourally, by administering a single injection of CCI-779 under light anaesthesia to the lumbar region of the spinal cord (L5 - L6 vertebral interspace) of SNL rats at day 14 post-surgery. After recovery, the rats were allowed to recover for 20 min and then tests involving the application of graded von Frey filaments (10 applications per paw) or acetone (5 applications per paw), were carried out every 20 min for 2 hr.

5.3.12 CCI-779 inhibits mechanical behavioural hypersensitivity from SNL rats

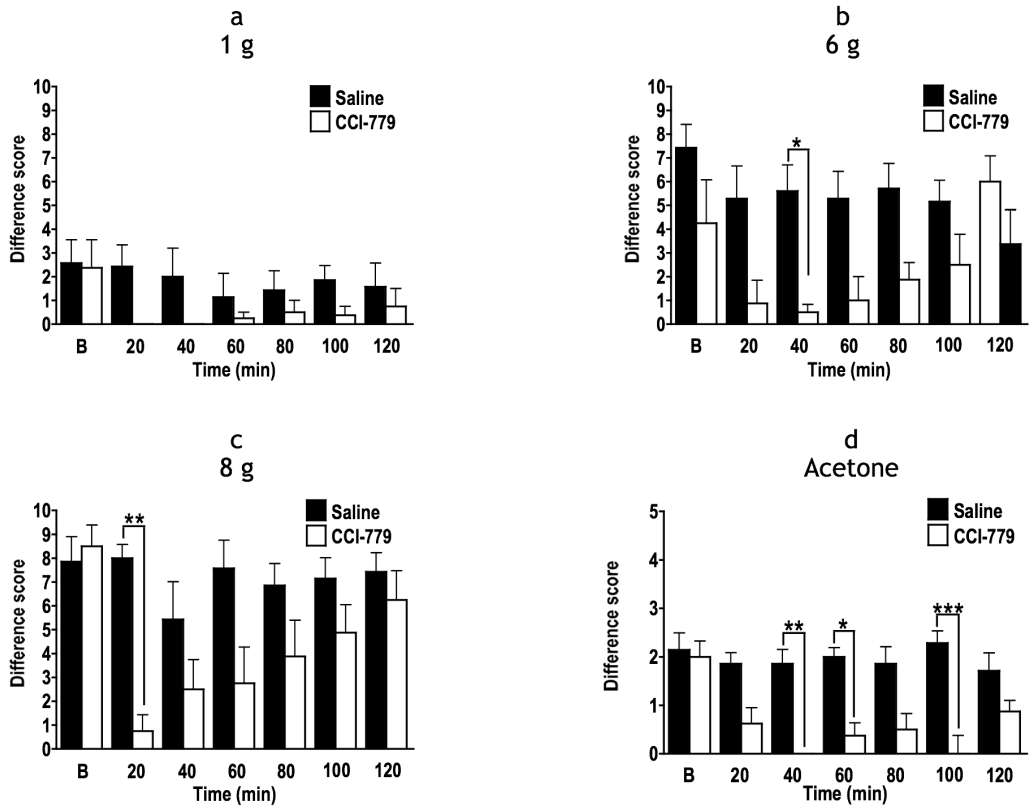


Figure 5.11 Effects of CCI-779 on behavioural hypersensitivity over 2 hr. (a) Trends for inhibition of withdrawal response difference scores by 250 μM CCI-779 (n = 8) compared to saline (n = 7) were seen as early as 20 min, with recovery to pre-drug baseline (B) levels. (b) CCI-779 inhibited withdrawal response difference scores to 6 g. Significance was achieved at 20 min where the difference score for saline treatment was 5.29 ± 1.37 and the difference score for CCI-779 treatment was 0.89 ± 0.97. (c) CCI-779 inhibited withdrawal response difference scores to 8 g. Significance was achieved at 20 min where the difference score for saline treatment was 8.00 ± 0.57 and the difference score for CCI-779 treatment was 0.75 ± 0.67. (d) CCI-779 inhibited withdrawal response difference scores to acetone application at 40, 60 and 100 min where the difference scores for saline treatment were 1.86 ± 0.29; 2.00 ± 0.19 and 2.29 ± 0.70 respectively. The difference scores for CCI-779 treatment were 0.00 ± 0.00; 0.38 ± 0.26 and 0.00 ± 0.36 respectively (*P<0.05; **P<0.01, ***P<0.001).

Much like the electrophysiological data, the behavioural data reveals that there is a greater degree of inhibition by CCI-779 taking place within the first hr in SNL rats and where these changes are not significant, there is a strong trend for inhibition. It was therefore decided to analyse AUC data for the first hr of testing. Statistically significant differences between saline and CCI-779 treatment groups were determined by applying the Mann-Whitney test.

5.3.13 CCI-779 inhibits cold and mechanical behavioural hypersensitivity from SNL rats

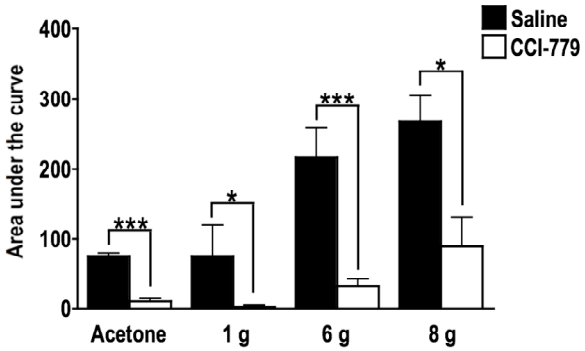


Figure 5.12 Effects of CCI-779 on behavioural hypersensitivity over 1 hr. Saline AUC of the difference scores for acetone, 1 g, 6 g and 8 g were 76 ± 4; 76 ± 43; 217 ± 41 and 268 ± 37 arbitrary units respectively. 250 μM CCI-779 AUC of the difference scores for acetone, 1 g, 6 g and 8 g were 10 ± 5; 3 ± 3; 33 ± 10 and 89 ± 41 arbitrary units respectively. For all data sets, saline treatment n = 7; 250 μM CCI-779 treatment n = 8 (*P<0.05; **P<0.01; ***P<0.001).

To summarise, the rapamycin analogue CCI-779 was effective in attenuating neuronal responses to electrical, mechanical and thermal stimuli. Like the naive rats in chapter 3, neuronal responses of SNL rats were attenuated in the second hr of testing. However, unlike naive rats, this attenuation was in fact greater in the first hr of testing, particularly for mechanical stimuli. When cross-comparing neuronal responses to CCI-779 within the first hr, it was apparent that this degree of inhibition was specific to SNL rats and not their sham counterparts. Also of interest was the fact that mechanically evoked responses and not thermally evoked responses from SNL rats were inhibited compared to shams and pre-drug controls. In addition, behavioural studies of SNL rats that had received CCI-779 revealed that the drug was also effective in inhibiting behavioural hypersensitivity to cold and mechanical stimuli as a result of SNL. Much like the electrophysiological data, it was found that the majority of the maximal inhibitory effects occurred within the first hr of administration of the drug. In addition, evoked responses after the maximal inhibitory effects of CCI-779 returned to pre-drug baseline values confirming the reversible nature of the drug (see figure 5.11).

5.4 Discussion

Chapter 4 examined the importance of rapamycin-sensitive pathways in the induction of formalin-induced inflammation in terms of neuronal hyperexcitability and behavioural hypersensitivity. In this chapter, the importance of rapamycin-sensitive pathways in a more persistent pain state- SNL-induced neuropathy was used to assess other aspects of this protein translation pathway. The neuropathic state induced by SNL is a much longer-lasting pain-like state and the long-lasting behavioural hypersensitivity to innocuous stimuli is in many ways similar to the allodynia presented by patients. Furthermore, whilst the formalin test was used to test the importance of rapamycin-sensitive pathways in the induction of a pain-like state, the SNL model was used to test the importance of rapamycin-sensitive pathways in maintaining pain-like states, by administering the rapamycin ester CCI-779 (in electrophysiological or behavioural studies) after the full establishment of the persistent pain-like state.

These studies show that the SNL model produces robust hypersensitivity to innocuous and cold stimuli of the innervated hind paw from as early as early as day 2 post-surgery and this is maintained for at least 2 weeks (see figure 5.1). In fact, this procedure has been found to produce long-lasting hypersensitivity to noxious heat for at least 5 weeks post-surgery and mechanical hypersensitivity at least 10 weeks post-surgery (Kim and Chung, 1992) perhaps mimicking chronic pain conditions in patients. The electrophysiological results clearly show that CCI-779 exerts a mixture of inhibitory effects in SNL rats when administered to the spinal cord. There appeared to be trends for a greater degree of inhibition by CCI-779 after the first test at 20 min or within the first hr of testing. This was particularly true for mechanically evoked responses to brush and 8 - 60 g von Frey filaments (see figures 5.2, 5.4 and 5.6). There were also significant inhibitions at later time points, but these changes were similar to those seen in Chapter 3 with naive animals, where many of the inhibitions exerted by CCI-779 occurred within the second hr of testing. This was particularly true for mechanical and thermal stimuli (see figure 3.7). Of interest here is that while the results in chapter 3 clearly show that there is an important role for rapamycin-sensitive pathways under physiological conditions, these pathways are also important for pathological

conditions such as persistent pain-like states. However, it appears that the changes that underlie persistent pain-like states may also change the activity of rapamycin-sensitive pathways to the extent where they are now more readily inhibited due to an increase in activity of these pathways.

CCI-779 was also tested electrophysiologically in sham rats. These rats had their nerves exposed but not ligated and therefore would be expected to produce a state similar to those of the naive rats in chapter 3. Although to some extent, this is true, it can be seen that the inhibitions seen during the second hr of testing in naive animals (see figure 3.7) are absent here, except for inhibitions by CCI-779 to 48 °C and 50 °C stimuli at 120 min (see figure 5.7). Furthermore, there are in fact facilitations of wind up responses at 40 min and also responses to 15 g at 80 min (see figures 5.3 and 5.5). Whilst these results clearly indicate that rapamycin-sensitive pathways are specifically altered in SNL rats, they also suggest that they are also altered in sham rats too. This is to be expected as sham surgery still involves disruption of tissue and stimulating the nerves albeit in a less physically damaging way than SNL surgery.

Due to the fact that CCI-779 appeared to have a more significant effect within the first hr of administration in SNL rats compared to sham and naive rats, the effects of CCI-779 on stimulus evoked neuronal activity was further investigated by analysing the maximal changes (positive or negative) within the first hr after CCI-779 administration. This reanalysis revealed just how much inhibitory action CCI-779 was exerting in SNL rats compared to sham rats and pre-drug control responses. The pre-drug control responses for both sham and SNL neuronal responses were pooled into one group in order to make comparisons with sham and SNL rats treated with CCI-779. Although it might be expected that the pre-drug baseline neuronal response levels would differ between the two groups, this was not the case here. This may be due to the fact that although neuronal excitability would be expected increase in SNL rats, there is also a reduction of peripheral input to the spinal cord due to the ligation procedure (see chapter 6) so that neuronal changes are only apparent due to differential responses to drug treatment.

When compared to sham rats, C-fibre mediated transmission onto WDR neurones was significantly inhibited, as was post discharge and wind up of WDR neurones. There was also significant inhibition of punctate mechanically evoked responses to von Frey filaments 8 – 60 g. However, this inhibitory action was not extended to thermally evoked stimuli (see figures 5.8 – 5.10) suggesting that rapamycin-sensitive pathways are more important for transmission mediated by particular sensory modalities.

The effects of CCI-779 were also examined behaviourally in SNL rats at day 14. Here, the rapid effects on withdrawal responses to mechanical and cold stimuli were even more apparent than the effects of the drug seen in the electrophysiological studies. In most cases, maximal inhibition of difference scores occurred at 20 min when compared to saline administration, except for acetone, where inhibition was significant at 40 min. Also, in most cases, inhibition returned to baseline levels, except for acetone, where there was a more sustained level of inhibition (see figure 5.11). Again, reanalysis of withdrawal responses in the first hr of CCI-779 administration by looking at the AUC for the results produced in the first hr revealed the large extent of rapid inhibition of withdrawal responses to innocuous cold and mechanical stimuli. It should be noted that this was not a sedative effect as all tests were performed on fully conscious and alert rats. Furthermore, there have been no reports of this dose of rapamycin or rapamycin analogues administered via the i.t. route having any sedative effects.

These results together with the results in chapter 4 show that rapamycin-sensitive pathways are not only important in the induction of persistent pain-like states, but also in the maintenance of persistent pain-like states too. Of particular interest is the fact that rapamycin-sensitive pathways are specifically altered after nerve injury which results in an enhancement in the efficacy time course of CCI-779. These results bear some similarities to those obtained in a study investigating the effectiveness of the anti-neuropathy drug GBP in attenuating neuronal responses of SNL rats (Suzuki et al., 2005). In this study, GBP was found to have no effect on neuronal responses in the absence of nerve injury so that in sham operated animals, GBP had no effect after administration on any neuronal responses to electrical, mechanical or thermal stimuli. However, after nerve injury, systemic

administration of GBP attenuated spontaneous spinal neuronal activity as well as neuronal responses to mechanical and thermal stimuli. Since GBP binds to a unique site on the $\alpha 2\delta$ subunit (Gee et al., 1996), this suggests an enhanced functional role of calcium channels and/or $\alpha 2\delta$ subunits after nerve injury, much like a proposed enhanced functional role of rapamycin-sensitive pathways and/or mTOR.

Suzuki et al., also demonstrated the importance of descending serotonergic facilitation from higher brain centres acting at spinal 5-HT3Rs in the induction and maintenance of neuropathy and this has also been confirmed in other studies (Suzuki et al., 2004b; Suzuki et al., 2005; Rahman et al., 2006). The results form Suzuki et al., whereby ondansetron is administered i.t. to neuropathic rats bears striking similarity to that seen in this chapter. Suzuki et al., showed that ondansetron produced robust inhibitions of the mechanically evoked responses in SNL rats compared to sham rats or pre-drug controls, suggesting an enhancement in the effectiveness of the drug after peripheral nerve injury (Suzuki et al., 2004b). However, although neuronal responses to thermal stimuli were attenuated by ondansetron, the drug's effects on this modality did not differ between SNL and sham rats. This similarity in results demonstrates that much like descending facilitatory action at spinal 5-HT3Rs, rapamycin-sensitive pathways are also enhanced after peripheral nerve injury and are particularly important for mechanically evoked responses. In accordance with this is the study carried out by Svensson et al. whereby ondansetron administered i.t. attenuated formalin-induced behavioural hypersensitivity in a manner not dissimilar to rapamycin (Svensson et al., 2006). The possibility exists therefore of an interaction between spinal 5-HT3Rs and intracellular rapamycin-sensitive pathways that act to induce and maintain persistent pain-like states.

6 Visualising rapamycin-sensitive pathways at the spinal level

6.1 Introduction

The results so far have shown that rapamycin-sensitive pathways at the spinal level are important in the induction and maintenance of neuronal hyperexcitability and behavioural hypersensitivity, which arise as a result of direct nerve injury or tissue damage. Immunohistochemistry is the only technique which can identify an antigen in its tissue or cellular location (Polak and Van Noorden, 2003). It was therefore decided that immunohistochemistry would be used to determine exactly where rapamycin-sensitive pathways are active and therefore where rapamycin exerts its action on mTOR.

6.1.1 Location of rapamycin-sensitive pathways

In terms of neuronal excitability, most of the immunohistochemistry studies investigating the cellular and subcellular distribution of rapamycin-sensitive pathways have been carried out on primary neurones in culture or hippocampal slices. In terms of the subcellular distribution of these factors in hippocampal neurones, it has been found that various components of rapamycin-sensitive pathways i.e. eIF4E and 4EBP are distributed in a punctate pattern. Furthermore, some of the immunopositive puncta correspond to synaptic regions as revealed by synapsin double labelling. Specifically, the immunoreactivity signals for rapamycin-sensitive components oppose and slightly overlap with the signals for synapsin-I which is a presynaptic protein as well as overlapping with postsynaptic domain protein 95 (PSD 95), therefore suggesting their localisation at postsynaptic sites. In addition, mTOR-positive regions have been found in the dendritic shaft in a diffuse pattern that extends into synaptic regions (Tang et al., 2002; Schratt et al., 2004). Interestingly, it appears that the distribution of these signals depends partly on their level of activation such that phospho p70S6K immunoreactivity is very low in dendrites of hippocampal neurones and is restricted mainly to the soma until an LTP-inducing stimulus is applied (Cammalleri et al., 2003), when dendritic immunoreactivity increases.

At the tissue level in hippocampal slices, immunostaining for various components of rapamycin-sensitive pathways including eIF4E, 4EBP, mTOR and phsopho p70S6K has revealed rapamycin-sensitive pathways to be active in the cell bodies of the CA1 region (stratum pyramidale) as well as the dendrites of the CA1 region (stratum radiatum). Furthermore, in accordance with studies on primary neurones, dendritic immunoreactivity was shown to increase after the application of LTP-inducing stimuli (Tang et al., 2002; Cammalleri et al., 2003; Tsokas et al., 2005; Tsokas et al., 2007).

Perhaps more relevant to my studies, immunohistochemical staining of skin sections from glaborous and adjacent hairy skin of adult rat hind paw has revealed that mTOR and phospho mTOR are extensively expressed in subsets of primary afferent sensory fibres. Specifically, mTOR appears to be restricted to fibres that do not cross the dermal-epidermal junction i.e. A-fibres. To further substantiate this, it has been found that all mTOR and phospho mTOR immunoreactivity overlaps with that of N52 (a marker for myelinated A-fibres). Furthermore, intraplantar injections of rapamycin were found to reduce the immunoreactivity of phosphorylated phospho p70S6K as well as reduce capsaicin-induced secondary mechanical hypersensitivity and mechanical hypersensitivity to pinprick stimuli in the SNI model, both features thought to be mediated by capsaicin-insensitive A-fibres (Jimenez-Diaz et al., 2008).

Although rapamycin-sensitive pathways have been well characterised in the peripheral nervous system at the level of the hind paw, there have been no studies to date that have investigated the location of rapamycin-sensitive pathways at the spinal level. In this chapter, immunohistochemistry techniques were used to study rapamycin-sensitive pathways in DRG and the spinal cord of rats that had undergone SNL.

6.2 Methods

6.2.1 Spinal nerve ligation

Surgery, electrophysiological and behavioural studies were performed as described in 2.4.

6.2.2 Immunohistochemistry

Standard immunohistochemistry and tyramide signal amplification were performed as described in 2.6.

Details of primary antibodies and relevant secondary antibodies are given in table 6.1. All concentrations were based on suggestions from colleagues who had previously used the antibodies, or by the suppliers themselves. In the case of phospho p70S6K, a dilution of 1:50 in PBS plus 0.2 % v/v triton X-100 and 0.1 % w/v sodium azide was used based on a study which probed for this protein in brain slices (Damjanac et al., 2008). This concentration was tested firstly on brain sections that were prepared in exactly the same way as the spinal cord and DRG sections in an attempt to replicate the results using a modified protocol before testing it on spinal cord slices. In order to confirm neuropathy in the sections being analysed, the markers glial fibrillary protein (GFAP) and CGRP were used to label glial cells and nociceptive primary afferent terminals containing CGRP respectively in the superficial dorsal horn as these components have been shown to be altered as a result of spinal nerve ligation (Zhuang et al., 2006; Kaku et al., 2007; Zheng et al., 2008). Other markers of interest that were also stained for included N52 (200 kDa neurofilament or NF200), a marker of A-fibres (Ma, 2001) and PKCγ, a marker of a subset of interneurones in the superficial dorsal horn (Polgar et al., 1999). Specific lumbar regions were identified using a low power microscope and comparing the structural morphology of the sections to a rat spinal cord atlas.

Primary antibody	Secondary antibody
Rabbit anti-phospho p70S6K Thr 389 (plus antigen unmasking and tyramide signal amplification steps- see 2.6.3), 1:50, Cell signalling, USA	Bioyinylated goat anti-rabbit Immunoglobulin G; 1:400; Vector laboratories Inc, USA
Rabbit anti-CGRP, 1:200, Sigma Aldrich, UK	Goat anti-rabbit Cyanine 3 (Cy3), 1:400, Stratech, UK
Rabbit anti-GFAP, 1:5000, Dako, USA	Goat anti-rabbit Cyanine 3 (Cy3), 1:400, Stratech, UK
Mouse anti-NF200 (anti-N52); 1:500, Sigma Aldrich, UK	Goat anti-mouse Cy3 1:400, Stratech, UK
Rabbit anti-PKCγ, 1:500, Santa Cruz, USA	Goat anti-rabbit Cy3, 1:400m, Stratech, UK

Table 6.1 Primary and secondary antibodies used to localise proteins of interest.

6.2.3 Statistical analysis

For quantification of spinal cord immunohistochemistry, regions of interest templates were set (one set of templates for the ipsilateral side and one set of templates for the contralateral side) and maintained for all sections throughout the analysis. Fluorescence levels were determined by calculating the mean grey value using Image J 1.38x (USA) and corrected for background levels. All values were then normalised to the side contralateral to the injury. For normalisation, all mean grey values were normalised to the highest mean grey value of the contralateral side for that region (total, medial or lateral). A Wilcoxon matched paired test was then used to compare the normalised ipsilateral mean grey values to the normalised contralateral mean grey values (*P<0.05; **P<0.01 ***P<0.001).

6.3 Results

6.3.1 SNL results in loss of input to the spinal cord at L5

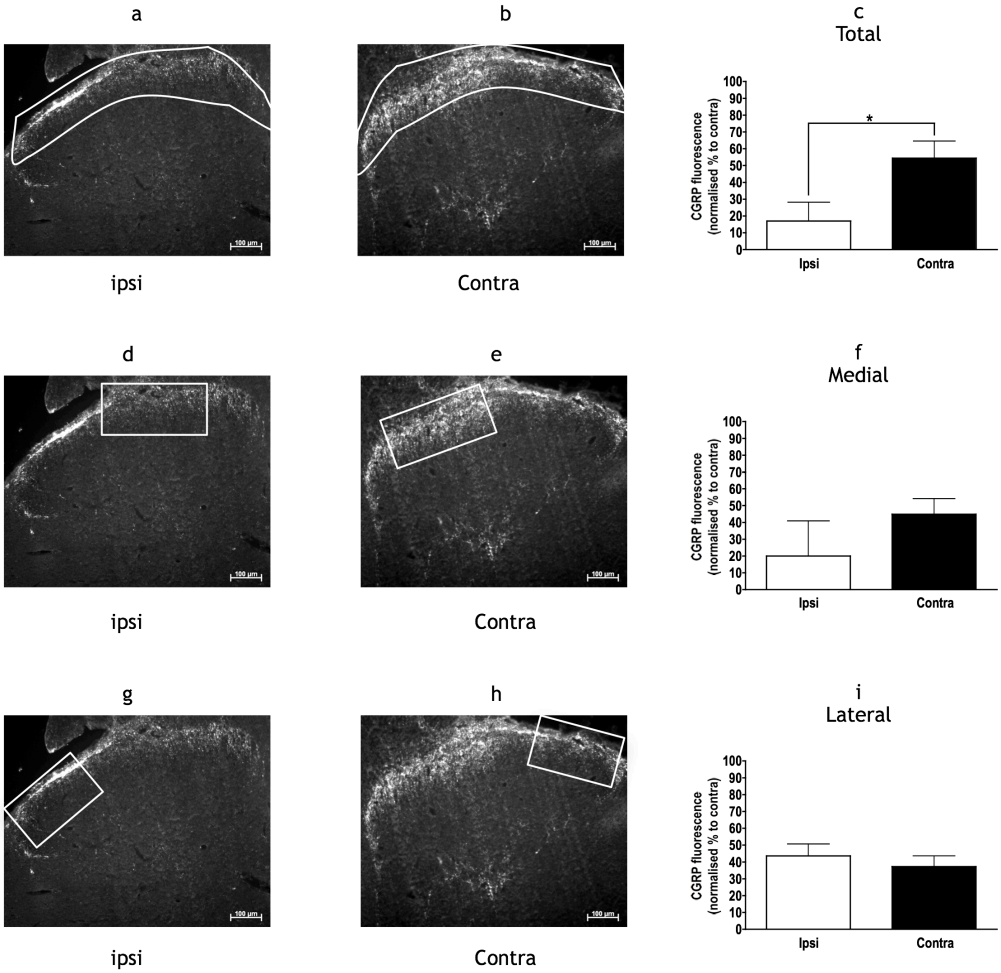


Figure 6.1 Effects of SNL on CGRP immunoreactivity in the spinal cord at L5. Immunostaining for CGRP in (a) the dorsal horn ipsilateral (ipsi) to the injury compared to (b) the dorsal horn contralateral (contra) to the injury was significantly lower when normalised to the contralateral side for the total region of interest. (c) CGRP fluorescence was found to be 17 $\pm$ 11 % in the ipsilateral side compared to 54 $\pm$ 10 % in the contralateral side. (d, e, f) There were trends for a reduction in input from the ipsilateral side compared to the contralateral side in the medial region of interest of the dorsal horn, which is at the dorsal root entry zone. (g, h, i) There were no significant differences between ipsilateral and contralateral immunoreactivity in the lateral region of interest of the dorsal horn. Scale bars = 100 μm. For all data sets, n = 12 sections from 3 rats (*P<0.05).

6.3.2 SNL has no effect on input to the spinal cord at L4

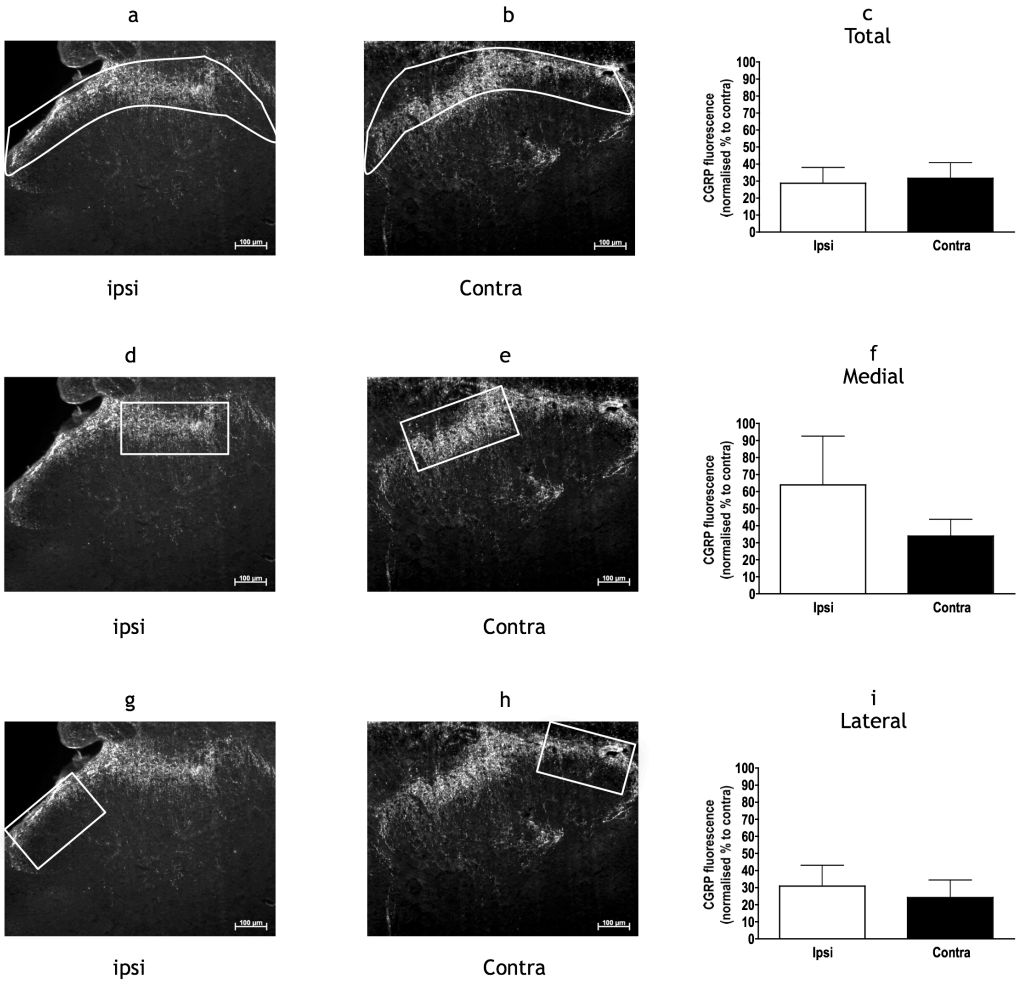


Figure 6.2 Effects of SNL on CGRP immunoreactivity in the spinal cord at L4. (a, b, c). There were no significant differences in immunoreactivity of the total region of interest between the dorsal horn ipsilateral (ipsi) and contralateral (contra) to the nerve injury. (d, e, f) Although there were trends for increases in immunoreactivity of the ipsilateral medial region of interest, these did not prove to be significant. (g, h, i) There were no significant differences in immunoreactivity between the ipsilateral and contralateral sites at the lateral region of interest. Scale bars = 100 μm. For all data sets, n = 9 sections from 3 rats.

6.3.3 SNL has no effect on input to the spinal cord at L3

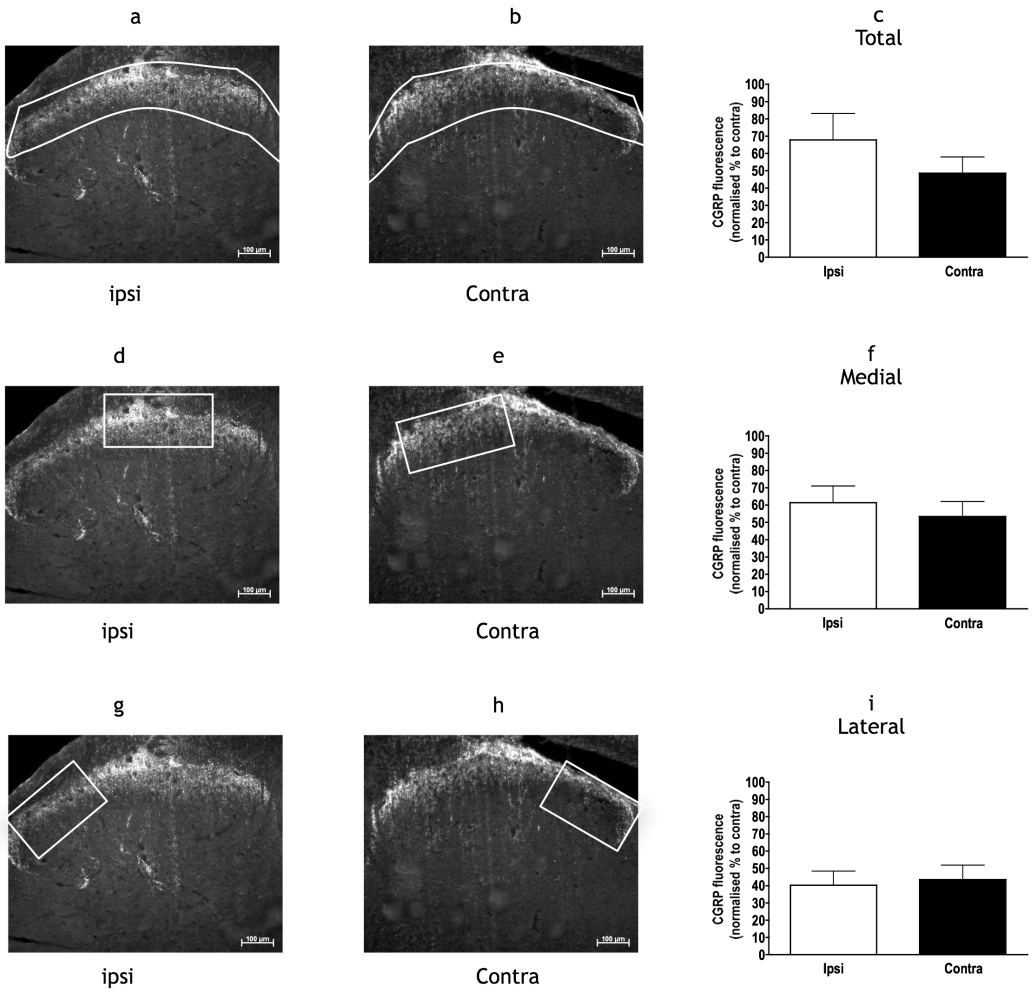
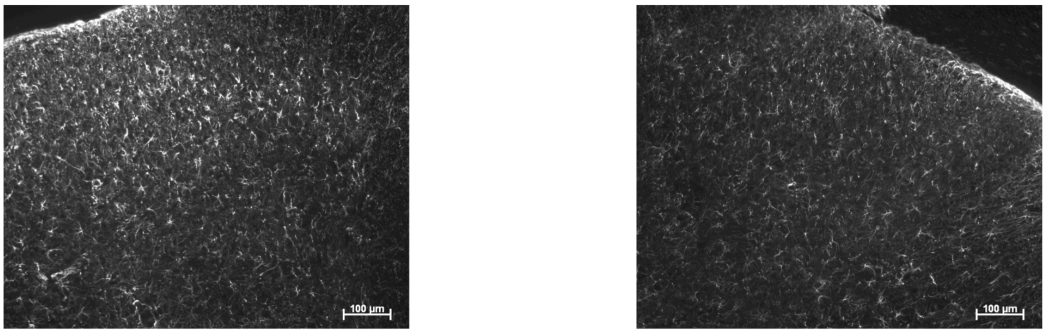


Figure 6.3 Effects of SNL on CGRP immunoreactivity in the spinal cord at L3. (a, b, c) There were no significant differences in immunoreactivity of the total region of interest, (d, e, f) the medial region of interest as well as (g, h, I) the lateral region of interest between ipsilateral (ipsi) and contralateral (contra) sites to the nerve injury. Scale bars = 100 μm. For all data sets, n = 9 sections from 3 rats.

6.3.4 SNL induces astrogliosis in the spinal cord



Ipsi **Contra**

Figure 6.4 Effects of SNL GFAP immunoreactivity in the spinal cord at L5. Staining with anti-GFAP to probe for astroglia shows a marked increase in immunoreactivity in the ipsilateral (ipsi) side compared to the contralateral (contra) side, which is a well established sign of nerve injury. Scale bars = 100 µm.

6.3.5 Establishing conditions for detecting phospho p70S6K

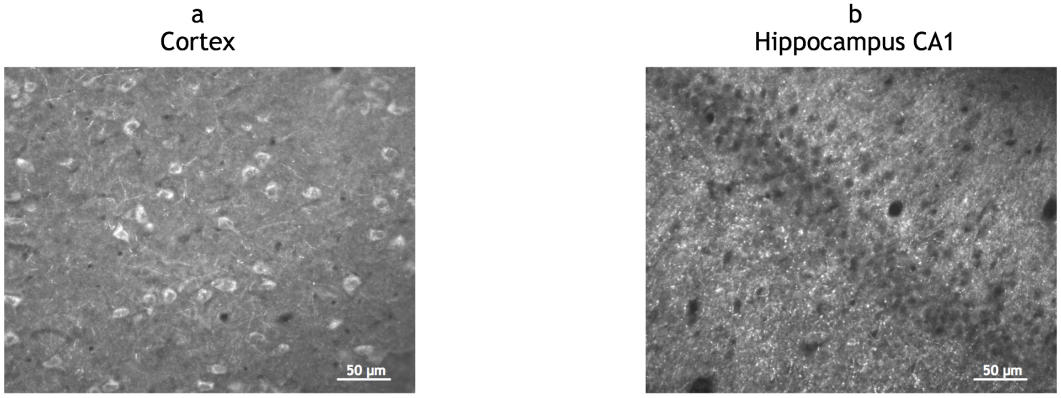


Figure 6.5 Phospho p70S6K immunoreactivity in cortical neurones and the CA1 of the hippocammpus. Since most immunohistochemistry studies probing for phospho p70S6K have involved using brain sections, here brain sections were used to validate the chosen protocol. Rabbit anti-phospho p70S6K was used to probe for phospho p70S6K using antigen unmasking and tyramide signal amplification steps. (a) Cortical neurones showing staining of the cell bodies as well as neuronal processes. (b) Hippocampal CA1 region showing some staining of the cell bodies (stratum pyramidale), but mainly staining of the dendrites (stratum radiatum) of the CA1 region. Scale bars = 50 μm.

6.3.6 SNL results in a reduction in phospho p70S6K activity at L5

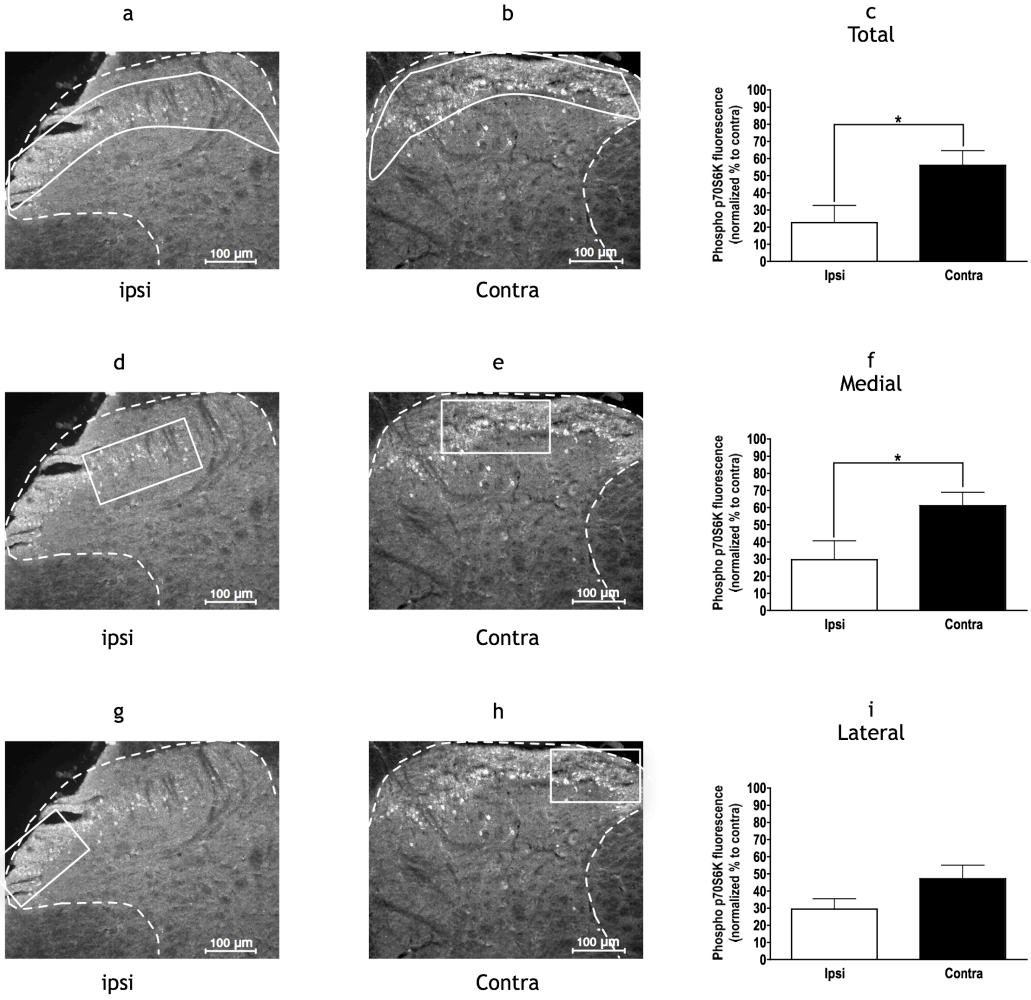


Figure 6.6 Effects of SNL on phospho p70S6K immunoreactivity in the spinal cord at L5. Immunostaining for phospho p70S6K in (a) the dorsal horn ipsilateral (ipsi) to the injury compared to (b) the dorsal horn contralateral (contra) to the injury was found to be significantly lower when normalised to the contralateral side for the total region of interest. (c) phospho p70S6K fluorescence was found to be 23 $\pm$ 10 % in the ipsilateral side compared to 56 $\pm$ 8 % in the contralateral side. (d, e, f) For the medial regions of interest, phospho p70S6K fluorescence was found to be 30 $\pm$ 11 % in the ipsilateral side compared to 61 $\pm$ 8 % in the contralateral side. (g, h, i) There were no significant differences between ipsilateral and contralateral immunoreactivity in the lateral regions of the dorsal horn. Scale bars = 100 μm. For all data sets, n = 11 sections from 3 rats (*P<0.05).

6.3.7 SNL has no effect on phospho p70S6K activity at L4

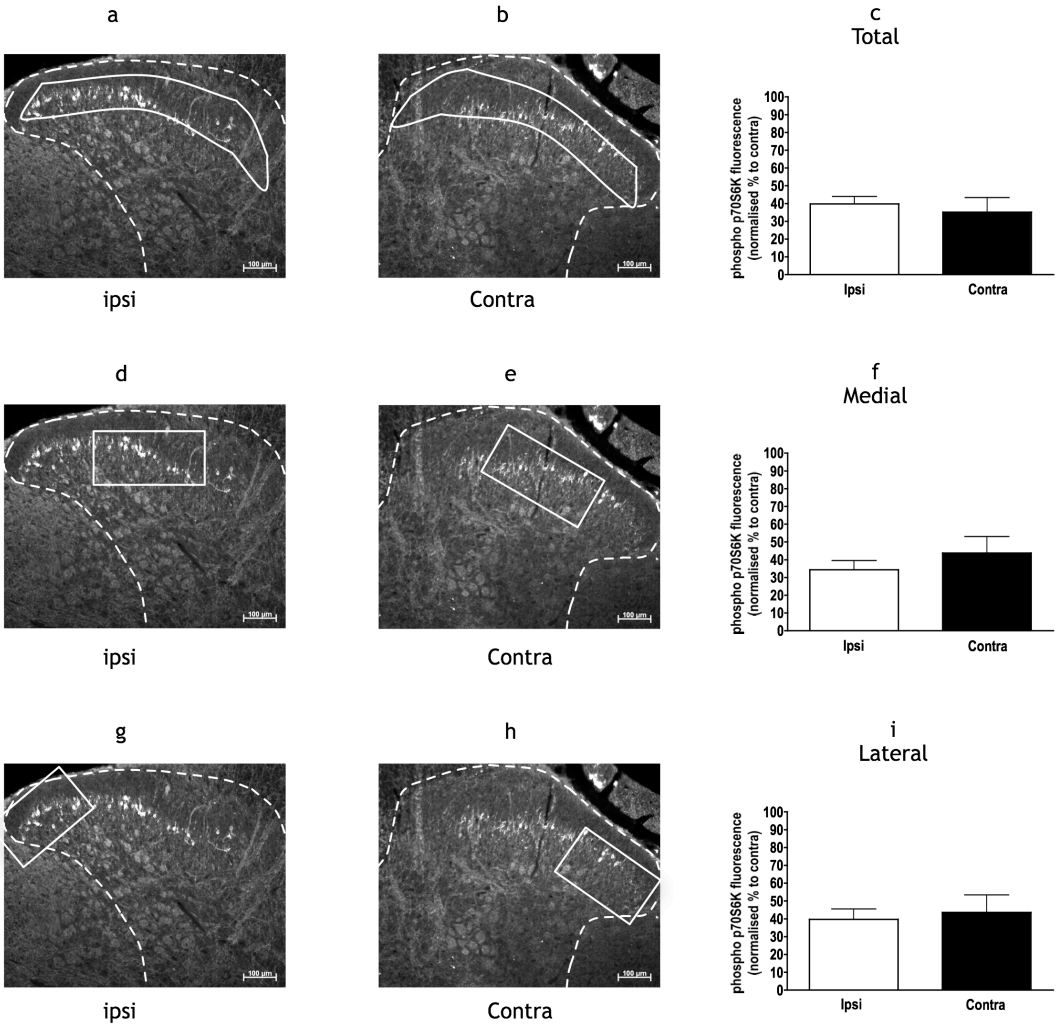


Figure 6.7 Effects of SNL on phospho p70S6K immunoreactivity in the spinal cord at L4. (a, b, c) There were no significant differences in immunoreactivity of the total region of interest, (d, e, f) the medial region of interest as well as (g, h, i) the lateral region of interest between ipsilateral (ipsi) and contralateral (contra) sites to the nerve injury. Scale bars = 100 μm. For all data sets, n = 12 sections from 3 rats.

6.3.8 SNL has no effect on phospho p70S6K activity at L3

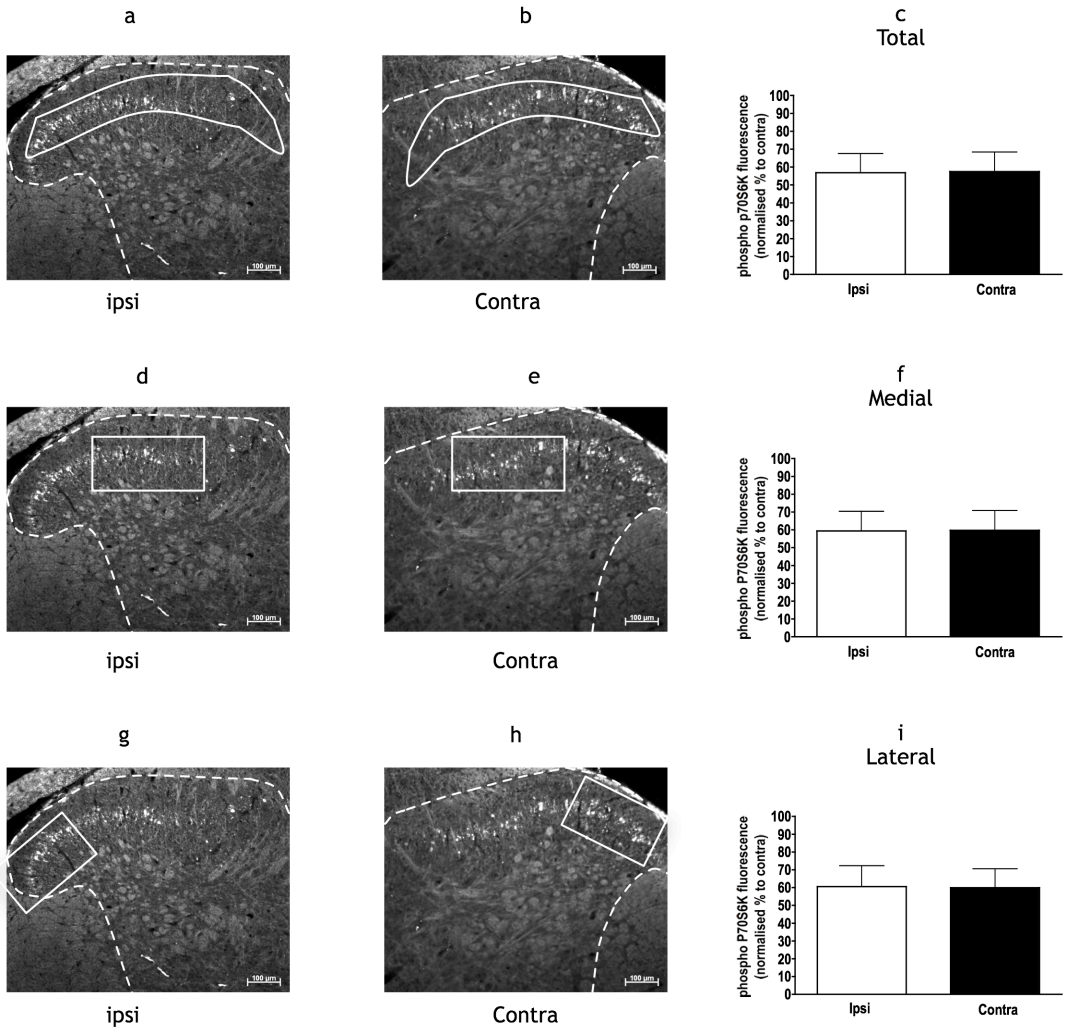
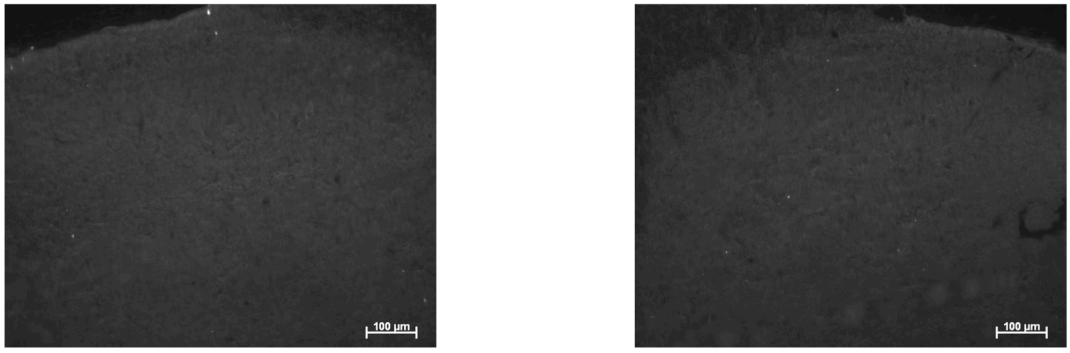


Figure 6.8 Effects of SNL on phospho p70S6K immunoreactivity in the spinal cord at L3. (a, b, c) There were no significant differences in immunoreactivity of the total region of interest, (d, e, f) the medial region of interest as well as (g, h, i) the lateral region of interest between the dorsal horn at ipsilateral (ipsi) and contralateral (contra) sites to the nerve injury. For all data sets, n = 11 sections from 3 rats. Scale bars = 100 μm.

In order to confirm specificity of the antibody against phospho p70S6K, control experiments were carried out whereby the primary antibody (anti-phospho p70S6K) was omitted from the protocol.

6.3.9 Anti-phospho p70S6K is antigen-specific



Ipsi Contra

Figure 6.9 Effects of the omission of the primary antibody on phospho p70S6K immunoreactivity. These sections were taken from L5 and the immunostaining protocol for phospho p70S6K was followed in exactly the same way as that used to obtain the previous results, except that the primary antibody was omitted from the protocol. This resulted in a complete loss of immunoreactive signal for phospho p70S6K in both the ipsilateral (ipsi) and contralateral (contra) sections. Scale bars = 100 µm.

Interestingly, immunoreactivity for phospho p70S6K was found to be restricted to a specific region of the dorsal horn of the spinal cord i.e. the inner layer of lamina II. This region is also known to contain a specialised group of small interneurones that stain positive for PKCγ and have been shown to be important in nociception and pain maintenance (Polgar et al., 1999). It was therefore decided to investigate whether neurones expressing phospho p70S6K were also positive for PKCγ.

6.3.10 Rapamycin-sensitive pathways are present in PKCγ-expressing neurones

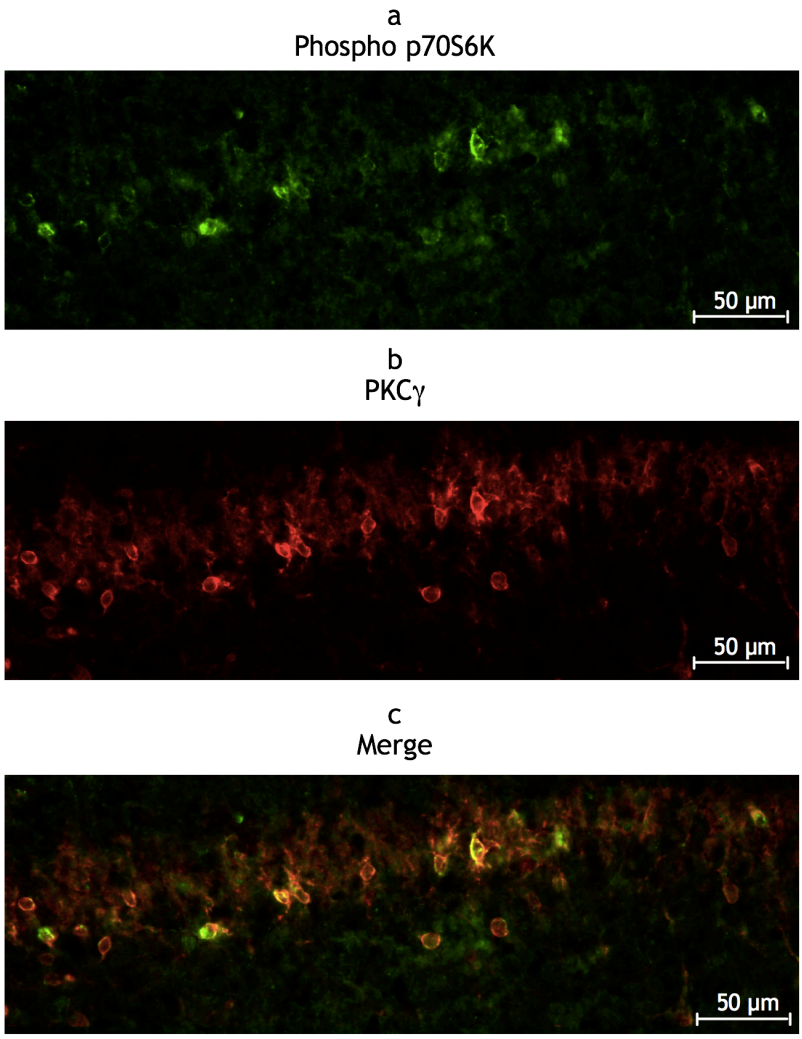
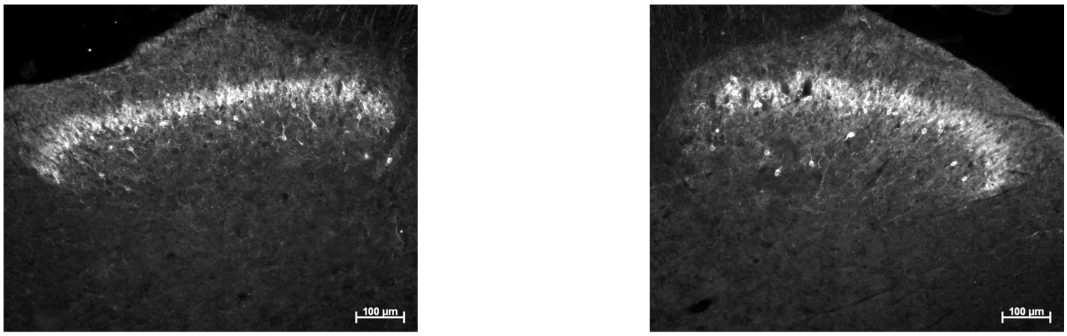


Figure 6.10 Ipsilateral dorsal horn inner lamina II neuronal immunoreactivity for phospho p70S6K and PKCγ. (a) Immunoractivity for phospho p70S6K shown in green. (b) Immunoreactivity for PKCγ shown in red. (c) Merged images show colocalisation of the two signals in orange. Scale bars = 50 µm.

Spinal cord sections at L5 were also stained solely for PKCγ to see if the immunoreactivity for this protein followed a similar pattern to the decrease in immunoreactivity for phospho p70S6K.

6.3.11 Immunoreactivity for PKCγ is unaltered at L5



Ipsi Contra

Figure 6.11 Dorsal horn immunoreactivity of PKCγ. Immunoreactivity of PKCγ unlike phospho p70S6K, is unaltered at L5 at dorsal horn sites ipsilateral (ipsi) or contralateral (contra) to the site of injury. Scale bars = 100 µm.

The role of rapamycin-sensitive pathways in the periphery at the level of the hind paw has been covered elsewhere (Jimenez-Diaz et al., 2008). However, what was not known was whether these pathways were also important in the periphery at the level of primary afferent terminals that are located within the spinal cord. In order to investigate this, DRG were sectioned and stained for phospho p70S6K in order to determine which fibre types and therefore which primary afferent terminals have the capacity for rapid protein translation.

6.3.12 Immunoreactivity for phospho p70S6K is present in DRG neurones

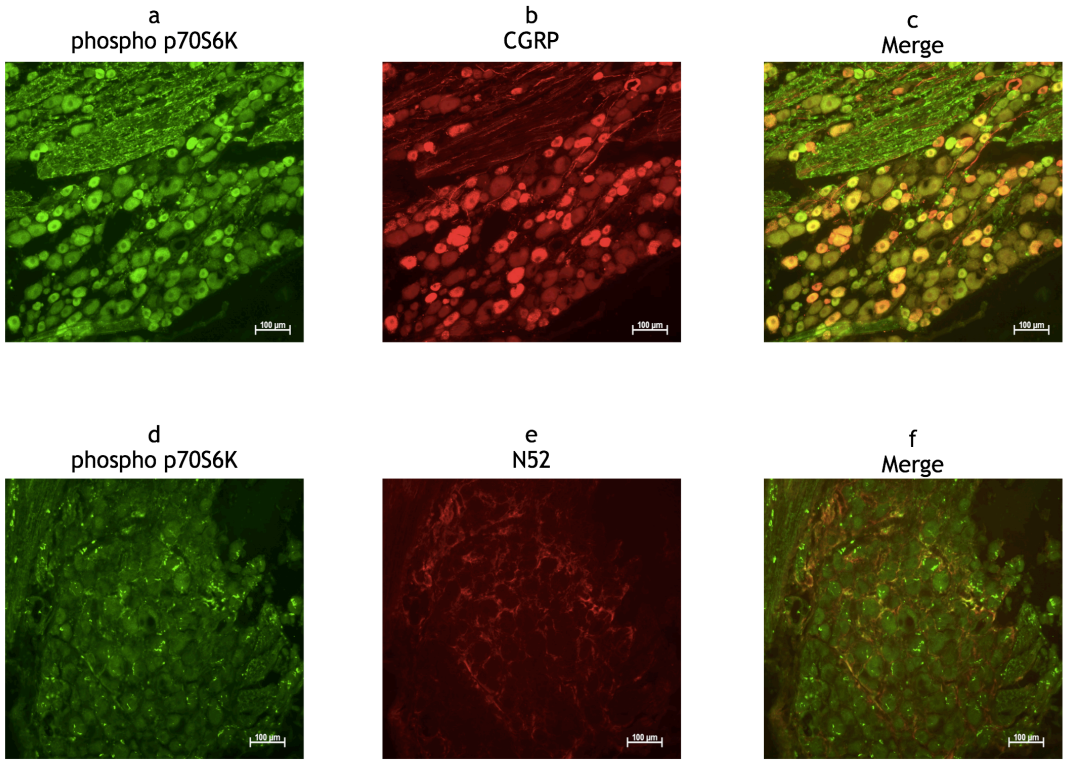


Figure 6.12 Phospho p70S6K immunoreactivity of C-fibres and A-fibres from DRG ipsilateral to nerve injury. (a) Immunoreactivity for phospho p70S6K shown in green. (b) Immunoreactivity for CGRP, a marker for small, unmyelinated C-fibres shown in red. (c) Merged images show colocalisation of phospho p70S6K and CGRP in C-fibres (in orange). It was not possible to determine colocalisation of phospho p70S6K and the marker for A-fibres (N52) using the protocol described in 2.6 (d, e, f). Similar immunoreactivity patterns were also seen in contralateral DRG (data not shown). Scale bars = 100 μm.

To summarise, SNL results in a loss of primary afferent input to the ipsilateral region of the spinal cord (L5) as shown by staining spinal cord sections with anti-CGRP. At L4 and L3, where the spinal nerves were uninjured, this change in immunoreactivity did not occur. SNL also results in astrogliosis in the same areas as shown by staining spinal cord sections with anti-GFAP. After validating the immunostaining protocol for phospho p70S6K in brain sections, immunostaining with anti-phospho p70S6K antibody in spinal cord sections ipsilateral to the nerve injury at L5 revealed that there was a decrease in immunoreactivity for phospho p70S6K. Again, this change in immunoreactivity was not seen at L3 and L4 where the spinal nerves were uninjured. Interestingly, immunoreactivity for phospho p70S6K was found to be restricted to the inner layer of lamina II of the dorsal horn and this is an area that is occupied by small interneurones that are immunopositive for PKCγ. Double labelling spinal cord sections with anti-phospho p70S6K and anti-PKCγ revealed that neurones positive for phospho-p70S6K appear to form part of a larger population of neurones that are PKCγ-positive. However, unlike the decrease in immunoreactivity for phospho p70S6K at dorsal horn sites ipsilateral to nerve injury, PKCγ immunoreactivity was unaltered. Finally, immunoreactivity for phospho p70S6K was examined in DRG as an indicator for possible activity in primary afferent terminals at the level of the spinal cord. Double labelling for phospho p70S6K with CGRP for small unmyelinated C-fibres revealed that rapamycin-sensitive pathways are present in many of these fibres, at least at the level of the DRG.

6.4 Discussion

The previous results chapters up to this point have clearly identified a role for rapamycin-sensitive pathways at the spinal level in the induction and maintenance of persistent pain-like states. However what was not known was where these pathways were active. This chapter set out to answer that question with the aid of immunohistochemistry techniques on spinal cord and DRG sections.

In this study, it was found that SNL resulted in a decrease in immunoreactivity in CGRP in superficial dorsal horn spinal cord regions ipsilateral to the site of injury (see figures 6.1 – 6.3). This finding is in accordance with that by Zheng et al. who found that following SNL, CGRP expression is not only reduced in the spinal cord, but it is also reduced in DRG for at least 28 days (Zheng et al., 2008). CGRP is therefore an excellent marker for confirming a neuropathic state at a purely cellular level. It is important to note however that a reduction in immunoreactivity is specific for SNL and other neuropathic models such as sciatic nerve crush and spinal nerve transection will actually cause an increase in immunoreactivity for CGRP in the spinal cord (Zheng et al., 2008). However, of importance here is that CGRP can be reliably used as a marker for neuropathy and its role as a multifunctional neuropeptide also means that these changes must at least in part contribute to the behavioural phenotype associated with neuropathic pain.

SNL also induces marked astrogliosis as shown by immunostaining for GFAP (see figure 6.4). This is an accordance with findings by Zhuang et al. who found that astrogliosis is present at 3 weeks post-SNL. This therefore not only implicates spinal astrocytes as having a potential role in maintaining persistent neuropathic pain, but also highlights the usefulness of GFAP as a marker for neuropathy (Zhuang et al., 2006).

Much like CGRP immunostaining, it was found that there was a decrease in immunoreactivity for phospho p70S6K in superficial dorsal horn spinal cord regions ipsilateral to the site of injury (see figures 6.6 – 6.8). This finding is of particular interest because perhaps intuitively, based on previous studies particularly concerning neuronal activity in the brain, a condition like neuropathy would be

expected to result in an increase in p70S6K immunoreactivity. For example, in hippocampal slices receiving high frequency stimulation in order to induce LTP, there is an increase in dendritic phospho p70S6K immunoreactivity (Cammalleri et al., 2003; Tsokas et al., 2005). In synaptosomes, phospho p70S6K immunoreactivity was shown to increase after treatment with BDNF (Takei et al., 2004). Furthermore, phospho p70S6K immunoreactivity has been shown to increase after learning in a fear conditioning paradigm (Parsons et al., 2006). Although, in a transgenic mouse model of Alzheimer's disease, immunoblotting for phospho p70S6K has revealed that there is less activity in these transgenic mutants compared to their WT littermates (Damjanac et al., 2008).

In terms of the results seen in this chapter, a decrease in immunoreactivity may mean that rapamycin and CCI-779, which are less active in naive animals now exert much more of an inhibitory action because there is less 'signal' to inhibit. This reduction in immunoreactivity presumably arises from a reduction of input to the spinal cord from primary afferent fibres, some of which are CGRP containing neurones, so it would be interesting to see how this result would vary depending on the neuropathic pain model that is studied. However, although it is clear that there is a reduction in immunoreactivity of phospho p70S6K, what is not clear is what role these rapamycin-sensitive pathways are playing in the neurones in which the activity remains. Is there an increase in turnover in pathway activity that contributes to neuropathy? Are these neurones inhibitory interneurones such that any decrease in activity in these neurones leads to disinhibition of a sensitised system and thus the pathophysiological changes associated with neuropathy? In addition, phospho p70S6K has also been identified in astrocytes albeit to a lesser extent than neurones (Damjanac et al., 2008). The focus of this study has been on neuronal activity, however, the contribution of non-neuronal cells to the various features of neuropathy have been well documented (see section 1.8)

The restricted localisation of phospho p70S6K is of particular interest in this study because these neurones are restricted mainly to the inner layer of lamina II and this is where neurones that show immunoreactivity for PKCγ also reside. Double labelling of sections for both PKCγ and phospho p70S6K revealed extensive overlap therefore confirming that phospho p70S6K-positive neurones also express PKCγ (see

figure 6.10). PKCγ is important because it has been shown that mice that lack the PKCγ isoenzyme fail to develop neuropathic pain-like syndromes and the neurochemical changes that occur in the spinal cord after nerve injury (Malmberg et al., 1997).

Besides being located in inner lamina II, PKCγ interneurones have also been shown to be present in lamina III, with some weak immunoreactivity in lamina I and the outer layer of lamina II. Most PKCγ-positive neurones are not GABA or μ-opioid receptor-immunoreactive (Polgar et al., 1999), therefore substantiating their roles as excitatory interneurones that have roles in the induction and maintenance of persistent pain-like states. It is important to note that expression of PKCγ,unlike phospho p70S6K did not appear to decrease (see figure 6.11). This suggests that PKCγ-positive neurones rely on input from primary afferent fibres in order to engage intracellular rapamycin-sensitive pathways.

Interestingly, each spinal segment receives nociceptive fibre inputs from several segmental dorsal roots. And it has recently been found that one third of lamina II interneurones receive simultaneous monosynaptic inputs from two to four different segmental roots. For lamina II interneurones, the major synaptic input has been found to be from the L4 - L6 roots, whereas for those located in L3, the input was from the L2 - L5 roots. Thus a new model is proposed whereby several C- or Aδ-fibres innervating one cutaneous region (peripheral convergence) and ascending together in a common peripheral nerve may first diverge at the level of the spinal nerves and enter the spinal cord through different segmental dorsal roots, but finally reconverge monosynaptically onto a single lamina II interneurone (Pinto et al., 2008). This model of organisation suggests that there will be changes seen in lamina II interneurones in L4 as a result of the loss of primary afferent input after SNL that is due to loss of inputs form L5 and L6. Yet, my results show that the reduction seen in denervated L5 and L6 in p70S6K are not replicated in L4, suggesting some upregulation in this signal in this segment (see figures 6.1 - 6.3 and 6.6 - 6.8), thus contributing to an increase in dorsal horn excitability and central sensitisation.

Initially, it was widely accepted that PKCγ interneurones in the spinal cord receive their input from unmyelinated, non-peptidergic, IB4-positive nociceptors (Snider and McMahon, 1998). However, there is now recent evidence to dispute this (Neumann et al., 2008). In their study, Neumann et al. showed that the terminal field of IB4-positive neurones in fact lies dorsal to that of PKCγ interneurones. In contrast, medium diameter and large diameter myelinated afferents that are generally associated with innocuous signal transmission and express the vesicular glutamate transporter VGluT1, were found to extensively overlap with PKCγ interneurones. Furthermore, PKCγ interneurones were specifically activated by innocuous input generated by rotarod-induced locomotion. These studies suggest that A-fibre mediated transmission should be affected by rapamycin and CCI-779, however, when observing the data in chapter 3 (see figure 3.2) and chapter 5 (see figure 5.8), this was not the case, as C-fibre mediated transmission was affected by these drugs to a greater extent than Aβ- or Aδ-fibre mediated transmission. This may be due to the fact that all fibres are stimulated at three times the C-fibre threshold during an electrical stimulus train. Whilst this means that nociceptive specific C-fibre wind up can be studied, it may also be the case that this stimulus is excessively suprathreshold for A-fibres and therefore that all A-fibre mediated changes are masked. However, even in SNL rats, where CCI-779 had a greater inhibitory effect on mechanically evoked responses, dynamic innocuous brush evoked responses and low mechanical stimuli below 8 g, which we can assume to be mainly mediated by A-fibres, were not affected by CCI-779 (see figures 5.9 – 5.10). My studies therefore suggest that the net effect of rapamycin and CCI-779 is an inhibitory effect on C-fibre mediated transmission onto WDR neurones and therefore noxious stimulus evoked responses.

Interestingly, CGRP nociceptors were found to be immunoreactive for phsopho p70S6K in DRG (see figure 6.12). This finding is at odds with the findings by Jimenez-Diaz et al., who found that in the periphery, at the level of the hind paw, all phospho mTOR and mTOR staining was found to co-exist with N52 immunoreactivity in A-fibres that do not penetrate the dermal-epidermal junction, indicating that mTOR is largely restricted to myelinated A-fibres (Jimenez-Diaz et al., 2008). This apparent contradiction may be due to a mismatch between

immunoreactivity in DRG and immunoreactivity at afferent terminals even though it is generally accepted that in most studied cases, the transport of molecules from DRG is in both directions to the periphery and along the central processes that terminate within the superficial dorsal horn. It is important to note however that Jimenez-Diaz et al. did find that rapamycin affected thresholds of some C-fibres confirming that rapamycin-sensitive pathways are also important in C-fibre mediated transmission. Furthermore, the mTOR binding protein raptor was found to be expressed in a small proportion of N52- negative fibres (Jimenez-Diaz et al., 2008).

Taken together, these studies suggest a link between primary afferent fibres, PKCγ and rapamycin-sensitive pathways. However, these are not the first studies to report links between PKC and rapamycin-sensitive pathways. In studies examining 5HT-induced long-term facilitation, phosphorylation of p70S6K and the rapamycin-sensitive increase in the synthesis and secretion of the sensory neurone neuropeptide sensorin has been shown to be dependent on PKC (Khan et al., 2001; Hu et al., 2007). Although PKC/PKCγ are clearly not the only upstream regulators of mTOR, these studies suggest a strong role for double labelled PKCγ-phospho p70S6K interneurones as well as primary afferent fibre terminals in the induction and maintenance of persistent pain states.

7 Rapamycin-sensitive pathways and upstream regulation by 5-HT

7.1 Introduction

Although the focus to this point has been on spinal mechanisms of pain maintenance, it is important to note that in these intact in vivo preparations, there will be an active component of descending modulation from the brain stem and higher centres in the brain that act on spinal neurones and peripheral afferent terminals, which also play an important role in pain maintenance. One major contributor to descending modulation of spinal neurones and primary afferent terminals is 5-HT. Relevant to pain processing, the predominant supraspinal source of 5-HT is the RVM, which can either enhance or dampen down incoming nociceptive signals from primary afferent fibres depending on the receptors that are activated (Millan, 2002).

7.1.1 5-HT-dependent neuronal hyperexcitability and behavioural hypersensitivity

With more than 15 receptor subtypes that are not solely confined to pain pathways, it is not surprising that 5-HT can exert a diverse range of physiological and pathological effects which are all dependent to some extent on serotonergic neurotransmission (Suzuki et al., 2004a). This diversity partly explains the conflicting views on the action of 5-HT at specific receptors as either a pro-nociceptive or anti-nociceptive transmitter. Serotonergic modulation of nociceptive processing in the spinal cord is mediated via the action of 5-HT on peripheral afferent fibres that terminate on neuronal cells, acting to either inhibit or facilitate neurotransmitter release onto spinal neurones; or by directly acting on serotonergic receptors on dorsal horn neurones themselves (Millan, 2002). Despite the diversity of serotonergic receptors and the opposing effects that the neurotransmitter may impart, dissecting out the serotonergic system in pain pathways has become a point of focus in understanding chronic pain conditions and more recently, there has been a greater emphasis on understanding the role of descending facilitation mediated by 5-HT.

5-HT is delivered to the spinal cord via the brain stem and higher centres in the brain (Millan, 2002). The obvious involvement of descending 5-HT pathways has, in recent times lead researchers to alter the activity of endogenous pathways in an attempt to further clarify the importance of 5-HT in maintaining persistent pain states. One research group has investigated this by administering the selective serotonergic neurotoxin 5,7 dihidroxytryptamine (5,7 DHT) i.t. to rats in order to deplete endogenous spinal 5-HT (Rahman et al., 2006). The effect of this procedure was examined in naive rats as well as rats that had undergone SNL. Electrophysiological studies of naive rats that had been given 5,7 DHT showed reductions in mechanically and thermally evoked responses of WDR neurones compared to rats given saline, suggesting that at the spinal level, 5-HT exerts a mainly facilitatory effect under physiological conditions. This effect was also seen behaviourally in rats that had undergone SNL after 5,7 DHT administration, i.e. SNL rats displayed attenuated responses to innocuous mechanical and cold stimuli compared to SNL rats that had received saline. These 5-HT associated changes were also independent of other spinal changes such as upregulation of NK1 receptors and microgliosis.

The studies by Rahman et al. suggest that supraspinal influences mediated by 5-HT are facilitatory and are responsible at last in part for the neuronal and behavioural changes associated with nerve injury-induced pain-like syndromes. Taking these studies a step further, another group have studied the pain-like behaviour of a conditional KO mouse (Lmx1b$^{f/f/p}$) which completely lacks 5-HT neurones in the CNS (Zhao et al., 2007). These mice were found to exhibit normal basal thermal sensitivity and visceral pain-like responses, although mechanical sensitivity was reduced compared to WT littermates. In the capsaicin-induced inflammatory pain-like model, there was an increase in the duration of mechanical behavioural hypersensitivity in these KO mice, which was reduced when these mice were administered 5-HT i.t. Furthermore, the analgesic effects of the antidepressants such as fluoxetine and amytriptilline were not as effective in the KO mice compared to the effects in WT mice, confirming a requirement of 5-HT for antidepressants to exert their pain-relieving effects These studies suggest that although the main role of the central 5-HT-mediated transmission in inflammatory hypersensitivity is inhibitory, its role in acute mechanical nociception is

facilitatory. This apparent contradiction in findings is not surprising and it is evident that different approaches that study different pain-like models or employ different methods and/or tools of altering the serotonergic system as well as studying it, will lead to different conclusions. What is undoubtedly true is that 5-HT is crucial to the modulation of nociception and persistent pain-like states.

Although the role of 5-HT in the induction and maintenance of persistent pain states is not clear-cut, recent studies have focussed on the role of the 5-HT3R, which, unlike all the other 5-HTRs, is the only ligand gated ion channel (Barnes and Sharp, 1999; Millan, 2002). 5-HT3Rs are located on the terminals of glutamate releasing myelinated primary afferent fibres as well as excitatory interneurones and some NK1 projection neurones in lamina I/III (Conte et al., 2005). As previously mentioned, 5-HT3Rs can be inhibited by the selective antagonist ondansetron. In the formalin test, pre-treatment with a single i.t. dose of ondansetron administered directly to the exposed spinal cord has been shown to attenuate neuronal hyperexcitability in both phases of the test, therefore highlighting the peripheral and central effects of 5-HT3R activation in neuronal hyperexcitability in a pain-like state (Green et al., 2000; Suzuki et al., 2002). In accordance with these studies is a study showing that ondansetron attenuates second phase formalin-induced behavioural hypersensitivity when administered 15 min prior to formalin injection (Svensson et al., 2006). Also, in SNL rats, a low dose of ondansetron which has no effects on neuronal responses from WDR neurones of sham rats, is effective in attenuating responses evoked by mechanical stimuli (Suzuki et al., 2004b). Furthermore, in a rat model of cancer-induced bone pain, the inhibitory effects of ondansetron i.t. on mechanically and thermally evoked responses are significantly greater compared to sham rats (Donovan-Rodriguez et al., 2006).

Substantiating the role of 5-HT3Rs in pain maintenance further, KO mice lacking the A subunit of the 5-HT3R, which is required for functionality of the receptor, have been shown to display normal acute pain-like responses, but ongoing hypersensitivity produced by formalin-induced inflammation is attenuated (second phase only) (Zeitz et al., 2002). Taken together, these results show that 5-HT facilitates persistent pain-like states via activation of 5-HT3Rs most likely due to an increased descending serotonergic drive from the brain and in particular, the

RVM (Suzuki et al., 2004a). In accordance with these findings, in a small randomised double-blind study, a single intravenous bolus of ondansetron has been shown to alleviate the overall pain experienced by neuropathic pain patients (McCleane et al., 2003).

Interestingly, not all pain models involve altered serotonergic activity at 5-HT3Rs. Carrageenan-induced inflammation has been shown to produce mechanical and behavioural hypersensitivity as well as significant neuronal plasticity (Kayser and Guilbaud, 1987; Stanfa et al., 1992). However, electrophysiological approaches have shown that when ondansetron is administered i.t. to rats with carrageenan-induced inflammation, stimulus evoked neuronal responses are inhibited to the same degree in both naive and carrageenan-injected rats (Rahman et al., 2004). Therefore, in this model, the spinal plasticity and the behavioural hypersensitivity are mediated by different mechanisms.

7.1.2 5-HT-dependent neuronal hyperexcitability and rapamycin-sensitive pathways

Studies on *Aplysia* have demonstrated that endogenous 5-HT is important for basic reflexes (Glanzman et al., 1989). In these studies *Aplysia* were treated with 5,7 DHT to deplete endogenous 5-HT which resulted in a reduction in the dishabituation (enhancement of responses after habituation) of the withdrawal reflex produced by tail shock as well as reduced plasticity of the siphon sensory neurones and corresponding motor neurones. These studies therefore indicate that 5-HT plays important roles in mediating dishabituation and sensitisation of the *Aplysia* withdrawal reflexes. In accordance with these findings, exogenous 5-HT has been shown to induce LTH of *Aplysia* neurones that can be blocked by prior treatment with rapamycin. Furthermore, localised 5-HT application to nerve segments will induce local axonal (and not somal) LTH that is inhibited by the 5-HT antagonist methiothepin (Casadio et al., 1999; Weragoda and Walters, 2007).

In an attempt to dissect out the components of the rapamycin-sensitive pathways that are important in contributing to 5-HT-induced LTH, Khan et al., examined the effect of rapamycin on the phosphorylation of p70S6K and found that p70S6K

phosphorylation was attenuated by rapamycin as well as an inhibitor of PKA (KT5720) and PKC (chelerythrine) (Khan et al., 2001). These results demonstrate that 5-HT can engage rapamycin-sensitive pathways and involves PKA and PKC. 5-HT has also been shown to decrease eEF2 phosphorylation, another downstream effector of mTOR that is inactivated by phosphorylation (Carroll et al., 2004).

The results from the previous chapters clearly demonstrate a link between persistent pain-like states and rapamycin-sensitive pathways. In addition, these persistent pain-like states are known to involve descending facilitatory serotonergic pathways mediated at least in part by 5-HT3Rs. The aim of this chapter was to investigate a possible link between 5-HT3Rs and rapamycin-sensitive pathways in rats using electrophysiological techniques. Specifically, the selective 5-HT3R antagonist ondansetron was used to block 5-HT3Rs and the selective 5-HT3R agonist 2-methyl-5-HT was used to activate these receptors. In addition, the carrageenan-induced inflammation model where descending serotonergic facilitation at 5-HT3Rs is not altered was also studied.

7.2 Methods

7.2.1 In vivo electrophysiology

See 2.2. All drugs were added i.t. to the exposed spinal cord in a volume of 50 μl. Tests were carried out every 20 min for 1 hr. In the studies using ondansetron to study the interactions between 5-HT3Rs and rapamycin-sensitive pathways, WDR neurones were first pre-treated with ondansetron (100 μg in 50 μl saline) or saline 10 min prior to rapamycin (250 nM or 11.43 ng in 50 μl saline/DMSO) which was also on the cord for 10 min prior to the first set of tests. In separate experiments, CCI-779 (250 nM or 12.88 ng in 50 μl saline) or a low dose of ondansetron (10 μg in 50 μl saline) was administered to the exposed spinal cords of rats with 2 % w/v carrageenan-induced inflammation (see 2.5) to determine if rapamycin-sensitive pathways are involved in this model of persistent hypersensitivity and also if serotonergic pathways which are affected by low dose ondansetron in neuropathic animals (Suzuki et al., 2004b) are also affected by low dose ondansetron in this model. Finally, to investigate the rapid mode of action of CCI-779, a facilitatory dose of the selective 5-HT3R agonist 2-methyl 5-HT (0.1 μg in 50 μl saline) was administered i.t. to rats after CCI-779 had been administered 2 hr earlier. For all these experiments, maximal changes in neuronal responses (positive or negative) over 1 hr were analysed.

7.2.2 Statistical analysis

Electrophysiological raw data are presented as mean $\pm$ SEM response (no. of spikes evoked by a given stimulus). When determining significant differences between neuronal responses for which there were only two groups, student's t-tests were used to compare differences in Aβ-, Aδ-and C-fibre firing, post discharge, input, wind up, brush and pin (unpaired for 7.3.2; paired for 7.3.4, 7.3.5 and 7.3.6). Two way ANOVA with repeated measures and Bonferroni post-tests were used to determine significance between groups for natural graded stimuli i.e. graded mechanical and thermal stimuli (*$P<0.05$; **$P<0.01$ ***$P<0.001$).

7.3 Results

7.3.1 Neurones selected for saline or ondansetron pre-treatment prior to rapamycin were not significantly different

	Saline (n = 7)	Ondansetron (n = 6)
Depth (μM)	620 ± 30	636 ± 27
Aβ-fibre threshold (mA)	0.87 ± 0.06	0.66 ± 0.11
C-fibre threshold (mA)	1.60 ± 0.18	1.23 ± 0.19
Aβ-fibre spikes	102 ± 25	154 ± 10
Aδ-fibre spikes	55 ± 15	70 ± 20
C-fibre spikes	235 ± 28	215 ± 38
Post-discharge spikes	140 ± 26	194 ± 64
Input spikes	304 ± 48	320 ± 64
Wind up spikes	158 ± 48	235 ± 84
Brush Spikes	336 ± 109	542 ± 77
1 g Spikes	46 ± 24	27 ± 18
6 g Spikes	256 ± 74	210 ± 48
8 g Spikes	360 ± 115	314 ± 49
15 g Spikes	457 ± 125	506 ± 75
26 g Spikes	593 ± 142	669 ± 70
60 g Spikes	707 ± 151	945 ± 95
35 °C spikes	248 ± 79	333 ± 93
40 °C spikes	313 ± 103	430 ± 94
45 °C spikes	547 ± 141	802 ± 95
48 °C spikes	724 ± 144	987 ± 122
50 °C spikes	858 ± 128	1120 ± 133

Table 7.1 Characterisation of WDR neurones selected for ondansetron or saline pre-treatment prior to rapamycin. For electrically evoked responses, a train of 16 pulses at three times C-fibre threshold (0.5 Hz, 2 ms pulse width) was applied to the corresponding hind paw. For naturally evoked responses, von Frey filaments or a small water jet was applied to the corresponding hind paw for 10 s. All data are expressed as raw mean values ± SEM.

7.3.2 Electrically and brush evoked neuronal responses show no link between spinal 5-HT3R activation and rapamycin-sensitive pathways

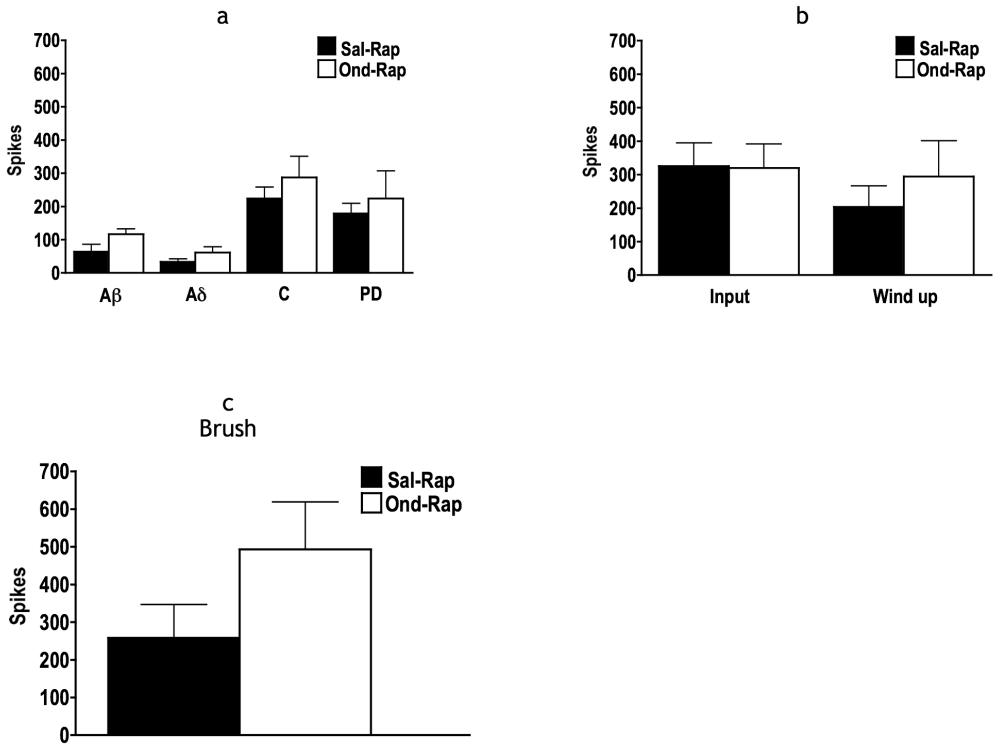


Figure 7.1 Effects of rapamycin on electrically and brush evoked neuronal responses after ondansetron and saline pre-treatment. (a) Aβ-, Aδ-, C-fibre mediated transmission as well as post-discharge (PD), (b) input and wind up (spikes after a train of 16 pulses) and (c) brush evoked activity (spikes during a 10 s stimulus) were not significantly different when the spinal cord was pre-treated with a high dose of ondansetron 10 min prior to rapamycin (Ond-Rap, n = 6) compared to when the spinal cord was pre-treated with saline 10 min prior to rapamycin (Sal-Rap, n = 7).

7.3.3 Spinal 5-HT3R activation due to noxious mechanically and thermally evoked neuronal responses engages rapamycin-sensitive pathways

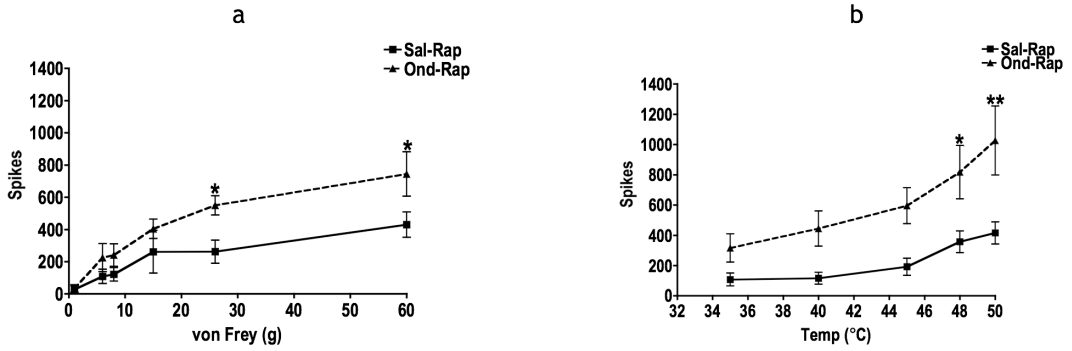


Figure 7.2 Effects of rapamycin on graded mechanically and thermally evoked neuronal responses after ondansetron and saline pre-treatment. (a) When the spinal cord was pre-treated with ondansetron 10 min prior to rapamycin, there was significantly less inhibition of noxious mechanically evoked responses compared to saline pre-treatment. Responses for ondansetron pre-treatment (Ond-Rap, n = 7) were 550 $\pm$ 60 and 744 $\pm$ 138 spikes for 26 and 60 g respectively. Responses for saline pre-treatment (Sal-Rap, n = 6) were 262 $\pm$ 60 and 430 $\pm$ 67 spikes for 26 and 60 g respectively. (b) When the spinal cord was pre-treated with ondansetron 10 min prior to rapamycin, there was significantly less inhibition of noxious thermally evoked stimuli compared to saline pre-treatment. Responses for ondansetron pre-treatment (Ond-Rap, n = 7) were 818 $\pm$ 176 and 1026 $\pm$ 228 spikes for 48 and 50 °C respectively. Responses for saline pre-treatment (Sal-Rap, n = 6) were 377 $\pm$ 60 and 416 $\pm$ 62 spikes for 48 and 50 °C respectively. Spikes = number of spikes during a 10 s stimulus (*P<0.05; **P<0.01).

These results seem to suggest that there is an interaction between 5-HT acting at 5-HT3Rs and rapamycin-sensitive pathways. In order to probe this interaction further, in vivo electrophysiology was used to study the effects of CCI-779 on inflammation induced by carrageenan, a model whereby at 3 hr, descending serotonergic pathways acting at 5-HT3Rs are unaltered (Rahman et al., 2004). In addition to these studies, the effects of a low dose of ondansetron that has been shown to be effective in pain-like states where descending serotonergic pathways acting at excitatory 5-HT3 pathways are active (Suzuki et al., 2004b) was studied in the carrageenan-induced inflammation model. Finally, the duration of suppression of rapamycin-sensitive pathways by CCI-779 was also tested by activating spinal 5-HT3Rs with the agonist 2-methyl 5-HT, which normally produces modest facilitations (Suzuki et al., 2005), after 2 hr of CCI-779 pre-treatment to confirm the long-lasting effects suggested in chapter 3.

7.3.4 CCI-779 has no effect on neuronal responses from rats with carrageenan-induced inflammation

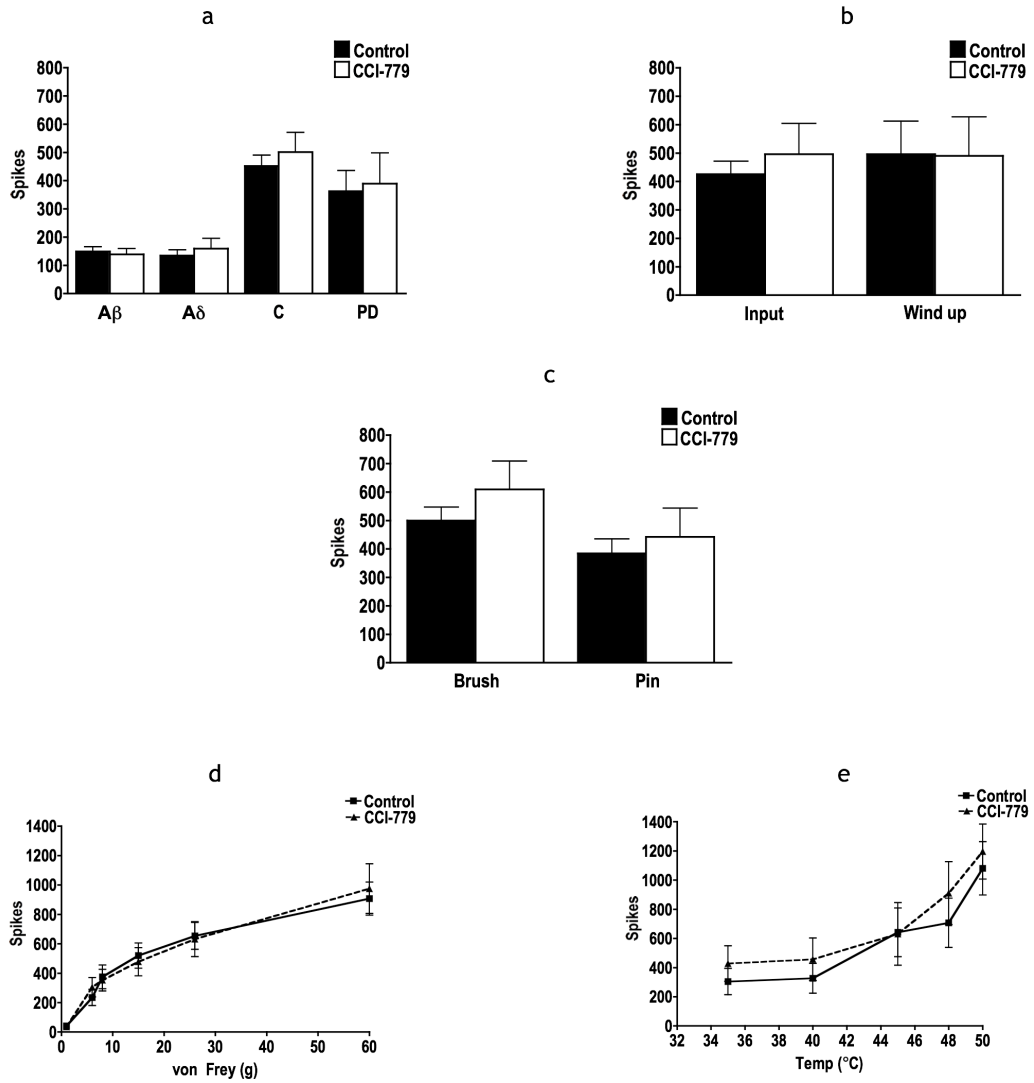


Figure 7.3 Effects of CCI-779 on neuronal responses from rats with carrageenan-induced inflammation. (a) There were no significant effects of CCI-779 on pre-drug control responses after carrageenan-imduced inflammation was established for electrically evoked Aβ, Aδ, C-fibre mediated transmission as well as post discharge (PD); (b) input and wind up (spikes after a train of 16 pulses); (c) brush and pinprick evoked responses as well as (d) graded mechanically and (e) graded thermally evoked responses (spikes during a 10 s stimulus). For all data sets, n = 10.

7.3.5 Serotonergic activity at spinal 5-HT3Rs is unaltered by carrageenan-induced inflammation.

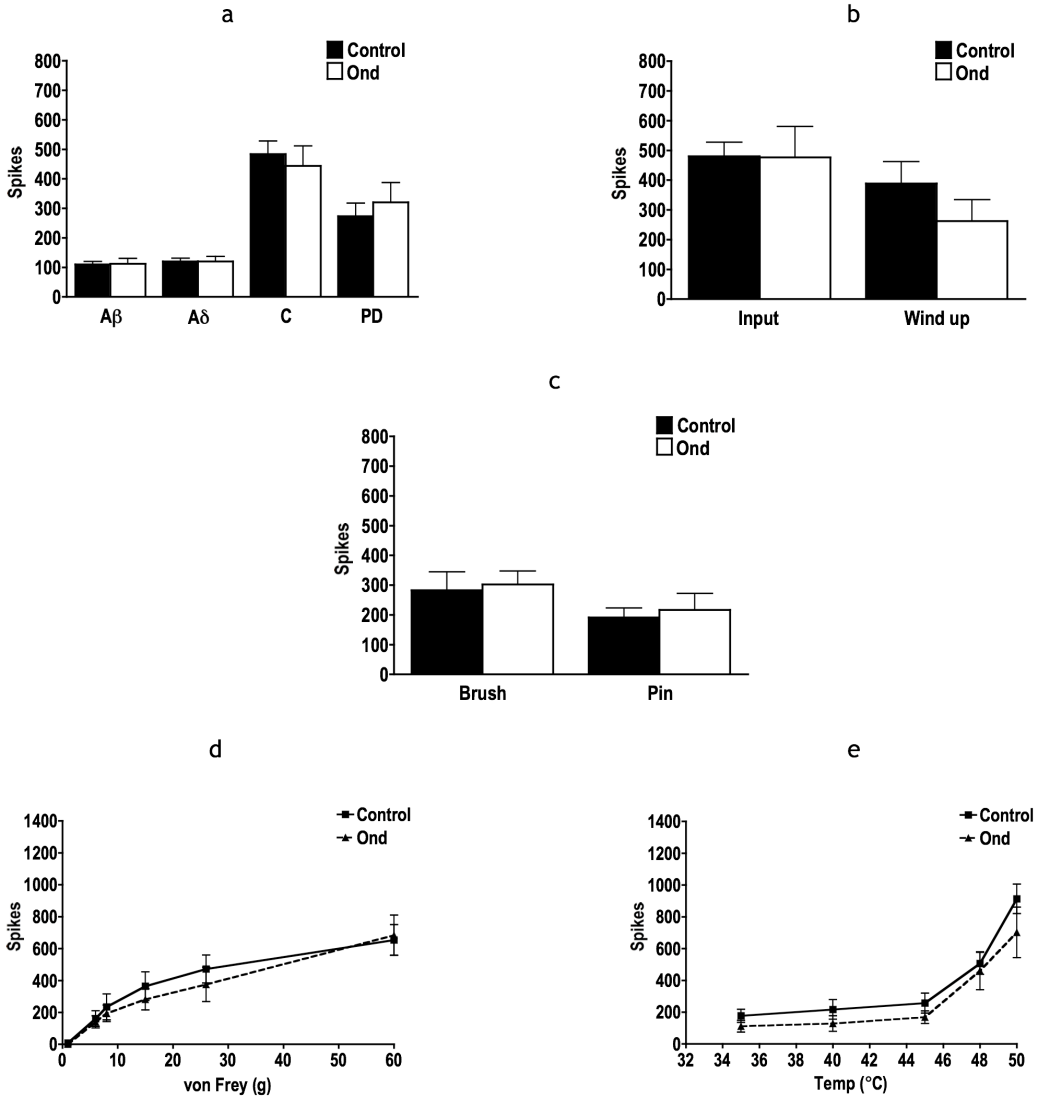


Figure 7.4 Effects of ondansetron on neuronal responses from rats with carrageenan-induced inflammation. (a) There were no significant effects of ondansetron on pre-drug control responses after carrageenan-induced inflammation was established for electrically evoked Aβ-, Aδ-, C-fibre mediated transmission as well as post discharge (PD); (b) input and wind up (spikes after a train of 16 pulses); (c) brush and pinprick evoked responses as well as (d) graded mechanically (e) graded thermally evoked responses (spikes during a 10 s stimulus). For all data sets, n = 9.

7.3.6 CCI-779 is still inhibitory at 2 hr post-administration

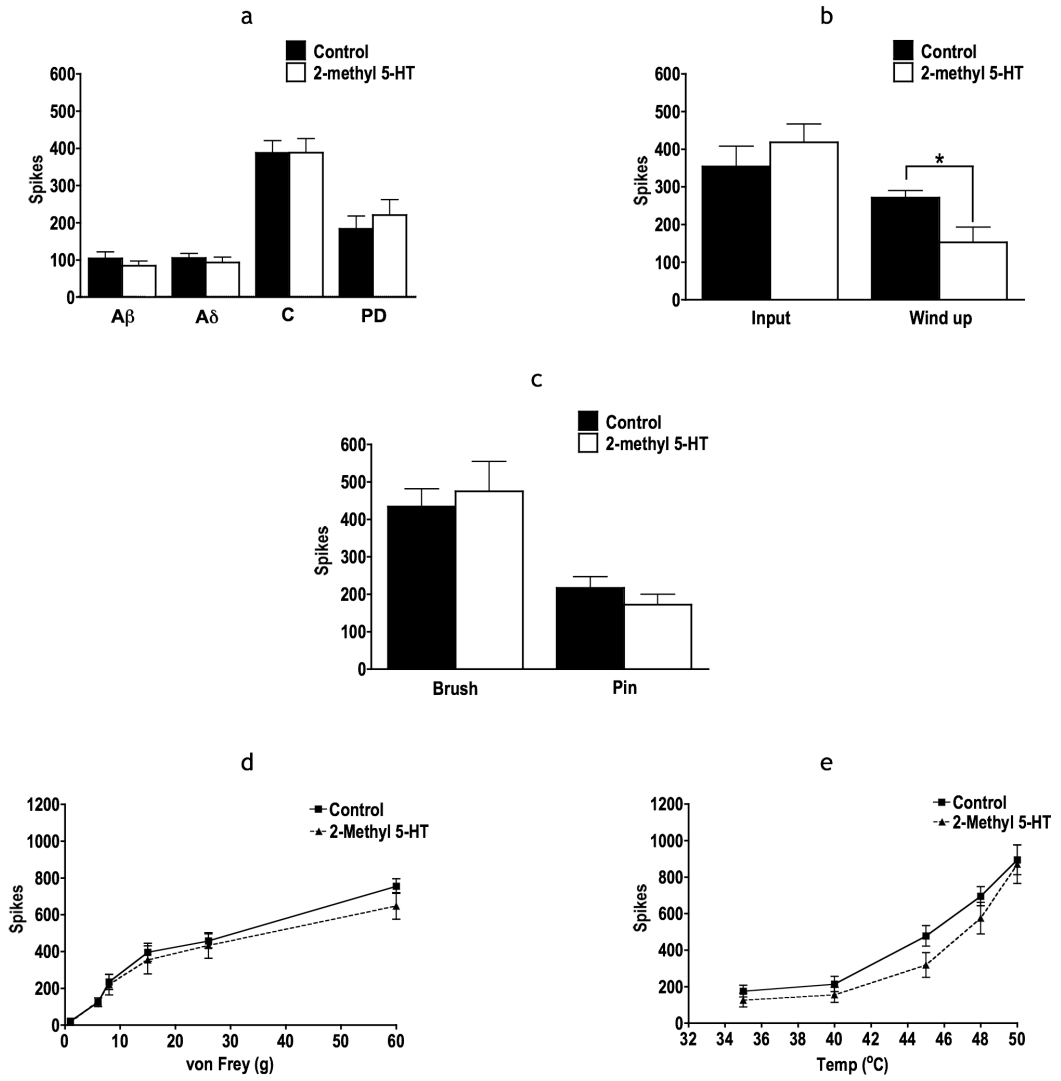


Figure 7.5 Effects of 2-methyl 5-HT administration on neuronal responses after 2 hr CCI-779 pre-treatment. (a) There were no significant effects of excitatory 2-methyl 5-HT on stimulus evoked responses after 2 hr CCI-779 pre-treatment for electrically evoked Aβ, Aδ, C-fibre mediated transmission as well as post discharge (PD). (b) Input was also unnaffected but wind up was significantly inhibited from 271 $\pm$ 19 to 153 $\pm$ 40 spikes (spikes after a train of 16 pulses). (d) Graded mechanically (e) graded thermally evoked responses were unaffected by excitatory 2-methyl 5-HT after 2 hr CCI-779 pre-treatment (spikes during a 10 s stimulus). For all data sets, n = 9.

To summarise, the inhibitory effects of rapamycin, were significantly reduced for graded mechanically and thermally evoked neuronal responses when the spinal cord was pre-treated with the selective 5-HT3R antagonist ondansetron. These results imply that descending serotonergic facilitation acting at 5-HT3Rs engage rapamycin-sensitive pathways. This was further substantiated in experiments examining rapamycin-sensitive pathways in a persistent pain-like state where descending serotonergic activity at 5-HT3Rs is unaltered i.e. carrageenan-induced inflammation. In these experiments, CCI-779 was ineffective in attenuating stimulus evoked neuronal responses. This was also the case with a dose of ondansetron that is known to have effects on stimulus evoked neuronal responses in SNL rats (Suzuki et al., 2004b). Finally, the time course of CCI-779 action was investigated by pre-treating the cord with CCI-779 for 2 hr prior to the addition of the facilitatory drug 2-methyl 5-HT. In these studies, CCI-779 was effective in attenuating wind up even 2hr after initial treatment with CCI-779.

7.4 Discussion

The results preceding this chapter showed that rapamycin-sensitive pathways were activated in persistent pain-like states that are known to involve activated descending serotonergic transmission that is facilitatory at spinal 5-HTRs. The aim of this chapter was to use pharmacological approaches to confirm that this was in fact true.

The selective 5-HT3R antagonist ondansetron has been shown to have inhibitory effects on stimulus-induced neuronal activity when administered i.t. in electrophysiological studies of persistent pain-like states (Green et al., 2000; Suzuki et al., 2002; Suzuki et al., 2004b; Suzuki et al., 2005). Interestingly, by pre-blocking 5-HT3R activation in naive animals, the inhibitory effects of rapamycin were reduced significantly for graded mechanically and thermally evoked stimuli, therefore suggesting that 5-HT3R activation engages rapamycin-sensitive pathways and also that descending facilitatory action of serotonergic pathways at 5-HT3Rs is permissive for the inhibitory action of rapamycin (see figures 7.1 and 7.2).

The permissive action by descending serotonergic activity at 5-HT3 receptors for the inhibitory action of rapamycin was further confirmed by two in vivo electrophysiology experiments utilising the carrageenan-induced inflammation model. Although this model has been shown to present behavioural hypersensitivity at 3 hr post carrageenan injection (Hedo et al., 1999) that is partly due to an increase in spinal inflammatory mediators such as prostaglandins (Yang et al., 1996), in vivo electrophysiology has revealed that in this pain-like state at this time point, descending serotonergic activity at 5-HT3Rs is unaltered (Rahman et al., 2004). The results from this chapter revealed that the rapamycin analogue CCI-779 had no significant effects on any stimulus evoked neuronal responses in rats where inflammation had been induced by carrageenan injection into the hind paw (see figure 7.3). This was also the case for a low dose of ondansetron (10 μg in 50 μl saline) which has been shown to be effective in attenuating stimulus evoked neuronal activity from rats with SNL (Suzuki et al., 2004b). Taken together, these results confirm that rapamycin-sensitive pathways are dependant at least in part upon descending serotonergic facilitation at 5-HT3Rs. It is important to note that

rapamycin-sensitive pathways could in theory, be activated by descending modulation at other excitatory or inhibitory receptors. However, persistent pain-like states involve shifts in neuronal thresholds and neuronal excitability to comparatively more excitatory states. Given what is already known about the importance of 5-HT3Rs in this process, excitatory 5-HT3R activation at the spinal level appears to be an important prerequisite for the activation of rapamycin-sensitive pathways.

Interestingly, although descending activation of spinal 5-HT3Rs is not altered at 3 hr, it may well be altered at a later time point. Indirect evidence of this comes from the study by Hedo et al. who showed that maximal behavioural hypersensitivity occurred at 3 hr post carrageenan injection. However, at 20 hr post carrageenan injection when thermal hypersensitivity fades and mechanical hypersensitivity remains, in an in vitro preparation using isolated spinal cord to measure spinal reflexes, it was found that there were significant increases in the responses to both single high intensity stimuli and to trains of stimuli, as well as a novel form of wind up that was induced by low intensity stimuli (Hedo et al., 1999). In addition, NMDA function is increased following 20 hr of carrageenan-induced inflammation (Rygh et al., 2001).

Mechanistically, although 5-HT has been shown to be important in engaging rapamycin-sensitive pathways (Casadio et al., 1999; Khan et al., 2001; Carroll et al., 2004; Weragoda and Walters, 2007), this the first study using these approaches to show specifically, that 5-HT3Rs are important upstream modulators of rapamycin-sensitive pathways. 5-HT3Rs are located on primary unmyelinated glutamatergic afferent terminals, excitatory interneurones as well as lamina I/III projection neurones (Zeitz et al., 2002; Conte et al., 2005). The results from chapter 6 show that rapamycin-sensitive pathways are present in spinal neurones and DRG and therefore likely to be present in afferent terminals, which has already been shown at the level of the hind paw (Jimenez-Diaz et al., 2008). Therefore, rapamycin-sensitive pathways may have different effects at different sites. At afferent terminals in the spinal cord, 5-HT3R activation would lead to an influx of Ca^{2+}, which could lead to activation of rapamycin-sensitive pathways via a Ca^{2+} dependent mechanism similar to NMDA mediated activation. These rapamycin-

sensitive pathways may contribute to glutamate release from nociceptive afferents into the spinal cord. Glutamatergic release into the dorsal horn of the spinal cord could then further activate and facilitate spinal neurones including excitatory interneurones. Activation of these excitatory interneurones via mGluRs and/or NMDARs engages rapamycin-sensitive pathways in spinal neurones that enhance dorsal horn neuronal excitability and these pathways may be more prominent particularly in persistent pain-like states.

The results in chapter 3 showed that in naive rats, CCI-779 has most significant inhibitory effects on stimulus evoked neuronal responses at >1 hr post i.t. administration (see figure 3.7). However, when rats had undergone SNL, the most significant inhibitory effects of CCI-779 were shifted to <1 hr. In this chapter, the selective 5-HT3R agonist 2-methyl 5-HT was used to mimic injury-induced activation of descending serotonergic activity at 5-HT3R (Suzuki et al., 2005). 2-methyl 5-HT was administered 2 hr after CCI-779 pre-treatment to investigate the probable time course of drug efficacy. Normally, 2-methyl 5-HT would be expected to cause modest facilitations of neuronal stimulus evoked responses. However, at 2 hr post rapamycin pre-treatment, these facilitations were absent and in the case of wind up, there was a significant inhibition when compared to pre-2-methyl 5-HT responses. These effects were likely due to the inhibitory action of CCI-779, confirming that CCI-779 can inhibit rapamycin-sensitive pathways for more than 1 hr even though the maximal effects are within the first hr of administration. However, determining if the immediate (<1 hr) actions of CCI-779 involve the same mechanisms as the later effects (>2 hr) would need further investigation. Importantly, these studies provide a link between peripheral activity, spinal cord excitability, descending serotonergic activity and rapamycin-sensitive pathways, providing new insights into the importance of protein translation mechanisms and persistent pain states.

8 Final discussion

Protein translation and therefore protein synthesis are physiological processes that are important to all living cells. Using rapamycin and the rapamycin ester CCI-779- two inhibitors of mTOR activity, I have shown that at the spinal level, protein translation that utilises mTOR is required for nociception and pain maintenance and can be engaged by descending pathways from higher brain centres.

8.1 Rapamycin-sensitive pathways are upregulated in persistent pain-like models

In vivo electrophysiology studies showed that in naive rats, rapamycin administered i.t. produced a dose-dependent inhibition of C-fibre mediated transmission to the spinal cord (see figure 3.2), as well as inhibition of mechanically evoked responses (see figure 3.3), although thermally evoked neuronal responses remained largely unaltered (see figure 3.4). Consequentially, in the formalin-induced inflammation model, when rapamycin was administered i.t. 3 min prior to formalin injected into the hind paw, there was a significant attenuation of the second phase of the formalin test. This was confirmed in behavioural studies where rapamycin was injected i.t. into lightly anaesthetised rats after which, they were allowed to recover before injecting dilute formalin solution into the hind paw. In these studies, rapamycin also inhibited behavioural hypersensitivity in both phases of the formalin test, therefore confirming a role for rapamycin-sensitive pathways in persistent pain-like states.

It would be logical to assume that if anaesthesia during the lumbar i.t. injection were not a factor, then the effects of rapamycin, like that in the electrophysiological studies would be seen as early as 3 min. However, the only feasible way to achieve this, whilst satisfying Home Office guidelines would be to implant a small polyethylene catheter through the atlanto-occipital membrane and down to the lumbar enlargement of the spinal cord. This procedure could be performed e.g. a week in advance of behavioural studies in order to allow for recovery from the procedure and would remove the need for anaesthesia during the administration of rapamycin to the spinal cord. However, whilst this is a useful

approach for studying the effects of repeated i.t. dosing over time, there are disadvantages associated with this technique. Specifically, this technique is associated with post-surgical mortality as well as neurological morbidity and the procedure can affect the overall functional state of the animal (Xu et al., 2006).

Whereas rapamycin was dissolved in 100 % DMSO before diluting with saline to achieve the appropriate concentrations, CCI-779 was dissolved in saline with the aid of a sonicator due to its improved solubility profile. It was however surprising that CCI-779 displayed a different response profile to that produced by rapamycin. Specifically, whereas rapamycin dose-dependently inhibited neuronal responses to electrical and mechanical stimuli in naive rats within the 1 hr time period allotted to each dose (see figures 3.2 and 3.3), as well as neuronal hyperexcitability and behavioural hypersensitivity due to formalin-induced inflammation (see figures 4.1 – 4.2 and 4.5 – 4.6), the effects of CCI-779 on neuronal responses from naive rats were delayed.

In electrophysiological studies, when a dose of CCI-779 equivalent to the top dose of rapamycin (i.e. 250 nM) was administered i.t. there were no inhibitions of C-fibre mediated transmission to the spinal cord (see figure 3.5). Furthermore, although mechanically and thermally evoked neuronal responses were attenuated, these effects were not present until the second hr of testing (see figure 3.7). Perhaps surprising however, was the finding that when CCI-779 was administered to nerve-injured rats, there was now a shift in the effectiveness of CCI-779 such that it was attenuating stimulus evoked neuronal activity within the first hr of testing, (see figures 5.6 and 5.8 – 5.10). However, changes in thermally evoked neuronal responses were still restricted to the second hr of testing. These results produced using electrophysiology techniques were substantiated by behavioural studies whereby CCI-779 injected i.t. into the lumbar region of the spinal cord attenuated nerve-injury induced behavioural hypersensitivity within the first hr of administration (see figures 5.11 – 5.12).

Taken together, these results suggest that whilst protein translation via mTOR is important under physiological conditions for sensory processing at the spinal level, protein translation via mTOR becomes more important under pathophysiological

conditions. This bears similarities yet also differences with previous studies investigating the importance of rapamycin-sensitive pathways in neuronal excitability. Previous studies demonstrated that whilst rapamycin was effective in attenuating hippocampal LTP, there were no effects on basal synaptic transmission (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005). In my studies, I have shown that as well as attenuating altered synaptic transmission in persistent pain-like states, rapamycin also inhibits sensory transmission at the spinal level in naive rats. It is important to note however that my experiments are conducted in whole animals rather than tissue slices and also that the mechanisms that mediate central sensitisation such as that observed in persistent pain-like states like formalin-induced inflammation and SNL are not necessarily identical to the mechanisms that mediate hippocampal LTP. In broad terms, central sensitisation and hippocampal LTP share two mechanisms- phosphorylation of synaptic receptors and insertion of new AMPA receptors into the postsynaptic membrane. However, whilst hippocampal LTP reflects only synaptic strengthening, central sensitisation also reflects other cellular mechanisms such as changes in intrinsic membrane properties and/or neuronal networks (e.g. disinhibition) (Ji et al., 2003).

8.1.1 Protein translation is rapidly inhibited by rapamycin and CCI-779

The results from chapter 4 show that rapamycin can attenuate formalin-induced neuronal hyperexcitability when injected i.t. as soon as 3 min prior to formalin being injected into the hind paw. Furthermore, in chapters 4 and 5 respectively, behavioural hypersensitivity due to formalin-induced inflammation and SNL is attenuated within 1 hr of rapamycin/CCI-779 administration. In the SNL studies, the attenuation of stimulus evoked neuronal responses as well as behavioural hypersensitivity was only temporarily inhibited after administration of CCI-779. This rapid yet temporary maximal effect of rapamycin and CCI-779 on neuronal responses and behavioural hypersensitivity indicates that rapamycin sensitive pathways are continuously active and that their continuous activation rapidly regulates neuronal activity. Such rapid signalling which has a direct effect on neuronal function is not a novel concept. It has been shown that NGF, which is implicated in peripheral sensitisation, can rapidly (< 10 min) lead to sensitisation of

TRPV1 receptors. This sensitisation involves PI3K-dependent mechanisms, phosphorylation of TRPV1 and increased insertion of TRPV1 channels into the surface membrane (Zhang et al., 2005). In addition and perhaps more relevant, the LTP-associated increase in the elongation factor eEF1A, which is downstream of mTOR, increases within 5 min after stimulation in a translation-dependent manner i.e. the increase is attenuated with the addition of rapamycin (Tsokas et al., 2005).

8.1.2 Rapid modulation of neuronal responses by rapamycin-sensitive pathways is not a feature of thermally evoked responses

It is clear that in persistent pain-like states, rapamycin-sensitive pathways are more important in mediating neuronal responses to electrical and mechanical stimuli rather than thermal stimuli. Figure 3.4 shows that inhibition of thermally evoked responses only occurs with the top dose of rapamycin and only at the lowest temperature tested (35 °C). Whilst rapamycin-sensitive pathways are important in mediating neuronal responses to thermal stimuli (see figure 3.7), the shift in response profile to CCI-779 observed with mechanically evoked neuronal responses after SNL was not apparent with thermally evoked responses (see figure 5.10).

This apparent selectivity may in part, be due to the classical 'heat sensor' TRPV1, which is known to play an important roles in pain processing and is activated by the chilli extract capsaicin. In a study examining the role of central and peripheral TRPV1 in pain processing, the antinociceptive effects of TRPV1 antagonists were compared in persistent pain-like states via different routes of administration (Cui et al., 2006). These drugs were known to have comparable in vitro potency, but differed in CNS penetration. When administered orally, both compounds effectively attenuated capsaicin-induced spontaneous hypersensitivity and CFA-induced thermal hypersensitivity. In addition, intraplantar or i.t. administration of the compound with good CNS penetration also attenuated CFA-induced thermal hypersensitivity, suggesting that both peripheral and central TRPV1 plays a role in inflammatory thermal hypersensitivity. When assessing markers of central sensitisation i.e. capsaicin- and CFA-induced mechanical hypersensitivity and

osteoarthritis-induced hypersensitivity, i.t. administration of the two compounds attenuated behavioural hypersensitivity to the same degree. However, when administered orally, the compound with good CNS penetration was much more potent, demonstrating that TRPV1 in the CNS plays a role in pain mediated by central sensitisation. Furthermore, referred or secondary mechanical hypersensitivity like that seen in the above persistent pain-like states is believed to be mediated by central sensitisation, whereas thermal hypersensitivity appears to be caused by peripheral sensitisation because it is restricted to the immediate area of injection (LaMotte et al., 1992). Thus, rapamycin-sensitive pathways may be less important in mediating thermally-evoked neuronal responses in persistent pain-like states because they are less important in peripheral sensitisation whereas they have a larger effect on mechanically-evoked responses in persistent pain-like states like SNL because they are more important in central sensitisation and may also be engaged by activation of TRPV1.

It is clear that there are many receptors that are at least to varying degrees, modality specific, yet many nociceptive fibres are polymodal (Julius and Basbaum, 2001). Despite this, there have been attempts to identify specific neurones and receptor subtypes that convey essential information sufficient to activate central pathways and elicit neuronal and behavioural responses relevant to pain. This is the case in a recent study whereby a thoroughly characterised Nav1.8 Cre recombinase-expressing mouse that shows normal pain behaviour was used to excise a floxed stop codon upstream of the globally expressed diphtheria toxin A (DTA)-subunit gene. Crossing the heterozygous Nav1.8 Cre mice with the homozygous DTA-expressing floxed mice resulted in control and DTA-expressing floxed mice in which all Nav1.8-expressing neurones (nociceptors) were killed. These mice showed attenuation of C-fibre-mediated activity, threshold and wind up; a loss of neuronal responses to mechanical stimuli but not thermal stimuli as well as behavioural hypersensitivity due to formalin-induced inflammation and CFA-induced inflammation and reduced sensitivity to cold. However, this genetic manipulation did not affect the mechanical and thermal hypersensitivity observed in nerve-injured mice. Taken together, the results demonstrate that Nav1.8-expressing neurones are essential for mechanical, cold and inflammatory sensation

but not neuropathic pain-like indications or heat sensing (Abrahamsen et al., 2008).

Although, parallels can be drawn between the response profile of Nav1.8-expressing neurones and rapamycin-sensitive pathways, it is clear from my research that unlike Nav1.8, rapamycin-sensitive pathways are important in neuronal and behavioural responses from nerve-injured rats (see chapter 5). However, the studies by Abrahamsen et al. suggest that there are perhaps specific pathways in the peripheral and central nervous systems that act to transmit modality and pain specific signals that may differentially engage rapamycin-sensitive pathways.

8.1.3 Rapamycin-sensitive pathways are engaged by descending facilitation acting at spinal 5-HT3Rs

A key feature of many persistent pain-like states and therefore central sensitisation is an enhanced drive from higher brain centres which converge on the RVM before projecting down to the spinal cord where they act mainly on 5-HT3Rs located on primary afferent terminals (Green et al., 2000; Suzuki et al., 2002; McCleane et al., 2003; Suzuki et al., 2004b; Conte et al., 2005; Suzuki et al., 2005; Donovan-Rodriguez et al., 2006). My studies have shown that rapamycin-sensitive pathways are important in mediating neuronal excitability and behavioural hypersensitivity in the formalin test (see chapter 4) as well as neuronal responses and behavioural hypersensitivity displayed by SNL rats (see chapter 5)- both models that are mediated by descending facilitation acting at spinal 5-HT3Rs. I have shown that rapamycin-sensitive pathways at the spinal level in the whole animal require descending facilitation for full activation. Specifically, noxious mechanically and thermally evoked responses that were inhibited by rapamycin, showed less inhibition when spinal 5-HT3Rs were pre-blocked with the selective blocker ondansetron (see figure 7.2). However, rapamycin-sensitive pathways were not affected in persistent pain-like states where descending facilitation acting at spinal 5-HT3Rs was unaltered i.e. carrageenan-induced inflammation, since the effects of CCI-779 were comparable to pre-drug control responses (see figure 7.3). A continuous serotonergic drive from the brainstem perhaps explains the continuous

nature of rapamycin-sensitive pathways as well as the rapid yet temporary action of CCI-779 with the studies focussing on SNL (chapter 4). I propose that with persistent pain-like states and particularly SNL, peripheral and central sensitisation result in a continuous drive from higher brain centres (particularly 5-HT from the RVM) which project down to the spinal cord. This continuous drive from higher centres sets in a motion an ongoing cycle of signalling involving mTOR, which acts to mediate neuronal and behavioural responses relevant to pain. These responses can be attenuated in the presence of rapamycin due to its inhibitory action on mTOR. Thus, when rapamycin or CCI-779 are diluted away from the site of action with the cerebrospinal fluid, the continuous drive from the RVM reengages rapamycin-sensitive pathways, which then continue to drive the neuronal responses and behavioural hypersensitivity associated with pain.

In the case of CCI-779, which shows minimal effects on neuronal activity from naive rats (see chapter 3), it is apparent that in order for the inhibitory actions of the drug to be revealed at the dose given, it must be administered to rats with an induced pain-like state that involves an increased serotonergic drive from supraspinal sites acting at spinal 5-HT3Rs. This state-dependency is similar to that seen in a study investigating mechanisms of action of the analgesic drug GBP (Suzuki et al., 2005). In this electrophysiological study, it was found that the usually powerful actions of GBP after SNL were blocked by either the ablation of NK1-expresssing neurones with i.t. SP-SAP, or the administration of i.t. ondansetron. However, activating spinal 5-HT3Rs with the selective agonist 2-methyl 5-HT, provided the state-dependency required for GBP to inhibit neuronal responses in uninjured rats. GBP binds to the $\alpha 2\delta$ subunit of VDCCs (Gee et al., 1996) so it is apparent that both 5-HT3R activation and $\alpha 2\delta$ subunit binding are prerequisites for GBP efficacy. Likewise 5-HT3R activation is a prerequisite for rapamycin/CCI-779 efficacy, yet it is extremely likely that there are other factors too due to what is known about the complexities of mTOR signalling (Hay and Sonenberg, 2004; Jaworski and Sheng, 2006; Swiech et al., 2008).

8.1.4 Rapamycin-sensitive pathways are present in nociceptors and spinal neurones

Using immunohistochemistry, I have shown that rapamycin-sensitive pathways are present in nociceptors at least at the level of the DRG and neurones of the spinal cord. A previous study has already demonstrated the presence of rapamycin-sensitive pathways in peripheral afferents at the level of the hind paw (Jimenez-Diaz et al., 2008). Specifically, components of rapamycin-sensitive pathways were present in fibres that terminate in the dermis and not the epidermis, a feature of myelinated A-fibres. The lack of C-fibre staining does not correlate with my results as I found that DRG staining revealed colocalisation between rapamycin-sensitive pathways and CGRP- a common marker of small unmyelinated fibres. I was not able to determine the presence of rapamycin-sensitive pathways in myelinated A-fibres that are associated with innocuous stimuli (see figure 6.12). Nevertheless, rapamycin-sensitive pathways may be present in all sensory fibres, at least in the DRG. Interestingly, Jimenez-Diaz et al. found that analysis of the mechanical thresholds of a small population of C-fibres revealed that they were actually influenced by rapamycin. Furthermore, the expression of the mTOR binding partner raptor was expressed in N52-negative fibres, thus substantiating my findings. It is possible that in the periphery, at the dermis/epidermis, whilst rapamycin-sensitive pathways are readily active in A-fibres, they are only activated in C-fibres after nerve injury/inflammation. It is important to note that the immunohistochemistry studies carried out by Jiminez-Diaz et al. were on naive rats and not rats in which a persistent pain-like state had been induced. There is also no reason why peripheral and central terminals should behave in exactly the same manner and there may be differential trafficking of proteins from the cell body.

To date, there have been no studies that have investigated the distribution of rapamycin-sensitive pathways in the spinal cord. Given what is known about the wide distribution of rapamycin-sensitive pathways and the wide range of processes that they mediate- not just limited to neuronal function, it was surprising that rapamycin-sensitive pathways in the spinal cord were largely restricted to a specific region in the dorsal horn of the spinal cord (see figures 6.6 – 6.8). Immunoreactivity for p70S6K appeared to be restricted to the inner layer of lamina

II. This was confirmed by double labelling for PKCγ due to the fact that PKCγ-expressing interneurones reside in this region of the dorsal horn (see figures 6.10 and 6.11). It had long been accepted that PKCγ interneurones in the spinal cord receive their input from unmyelinated, non-peptidergic, IB4-positive nociceptors (Snider and McMahon, 1998). However, in a recent study, it has been shown that in the spinal cord, PKCγ immunoreactivity overlaps with large myelinated fibre terminals that express VGluT1 and not IB4-binding nociceptor terminals. In addition, PKCγ interneurones were shown to be activated by innocuous input generated by rotarod walking (Neumann et al., 2008).

PKCγ interneurones have been shown to be important in pain processing since mice that lack the PKCγ isoenzyme fail to develop nerve injury-induced hypersensitivity (Malmberg et al., 1997). In addition, these interneurones are believed to be excitatory interneurones since the majority of PKCγ interneurones lack GABA or μ-opioid receptor immunoreactivity (Polgar et al., 1999). Taken together, it is apparent that mTOR signalling at several sites within the spino-bulbo-spinal loop mediates sensory transmission and therefore perception. I propose that rapamycin-sensitive pathways present in all sensory fibres mediate transmission to spinal neurones before the signals are then transmitted to higher brain centres. In the spinal cord, rapamycin-sensitive pathways are restricted to spinal PKCγ interneurones that receive input directly from myelinated A-fibres or indirectly from C-fibres that terminate in superficial laminae. These neurones consequently increase excitability of all dorsal horn neurones including the WDR neurones that are recorded in the electrophysiology studies. During peripheral and consequent central sensitisation, these pathways are upregulated, thus contributing to the neuronal and behavioural responses associated with pain-like states.

Perhaps surprisingly, analysis of p70S6K immunoreactivity revealed that there was a decrease in immunoreactivity on the injured side of the spinal cord compared with the uninjured side, a change that was restricted to the spinal segments innervated by the ligated nerves (see figures 6.6 – 6.8). This decrease in immunoreactivity was similar to that seen with primary afferent terminals that release CGRP (see figures 6.1 – 6.3) and represents the loss of primary afferents due to nerve damage. Interestingly, PKCγ immunoreactivity was unaffected by SNL

(see figure 6.11). This implies that rapamycin-sensitive pathways in PKCγ interneurones are activated by incoming signals from primary afferents. Therefore, I propose that despite the downregulation in p70S6K in denervated spinal cord segments, rapamycin-sensitive pathways must contribute to the abnormal evoked responses seen after peripheral nerve injury, which are conveyed by the intact or spared afferent fibres and are inhibited by both rapamycin and CCI-779.

8.1.5 mTOR: upstream neurotransmitters, downstream effectors

Taken together, my studies show that rapamycin-sensitive pathways are important in mediating neuronal activity in primary afferents and spinal neurones. Given what is already known about the spino-bulbo-spinal loop and neurotransmitters that activate rapamycin-sensitive signalling, I have outlined below different mechanisms of mTOR activation and its downstream effects.

5-HT

In Chapter 7, I described how the inhibitory effects of rapamycin were reduced in the presence of ondansetron (see figure 7.2). In addition, formalin-induced inflammation and SNL are pain-like states that are regulated by spinal 5-HT3Rs (Green et al., 2000; Suzuki et al., 2002; McCleane et al., 2003; Suzuki et al., 2004b; Conte et al., 2005; Suzuki et al., 2005; Donovan-Rodriguez et al., 2006; Svensson et al., 2006). Virtually all spinal 5-HT originates from the brainstem (Millan, 2002). I propose that central sensitisation is partly mediated by descending serotonergic pathways acting at spinal 5-HT3Rs located on peripheral primary afferent terminals of Aδ- and C-fibres, most of which lack SP (Kia et al., 1995; Zeitz et al., 2002; Conte et al., 2005). In primary afferents, rapamycin-sensitive pathways are activated via the Ca^{2+}-dependent recruitment of the GTPase Ras and canonical PI3K signalling pathways as well as other signalling pathways (see figure 1.4). These rapamycin sensitive pathways act to increase neurotransmitter release into the dorsal horn of the spinal cord perhaps via modulation of key downstream proteins and finally ion channel modulation. It is important to note that despite the conclusive evidence regarding 5-HT3Rs and their role in facilitating pain states, that there are other 5-HTRs located on primary afferent terminals that act to

modulate nociceptive signals at the spinal level and that can engage rapamycin-sensitive pathways. Yet these are GPCRs and so the effects of their activation can be just as antinociceptive as they are nociceptive and perhaps not as clear-cut as the excitatory 5-HT3R, which is an excitatory ligand-gated ion channel (Millan, 2002). However, it is clear that the same principles that apply to spinal 5-HT3Rs probably apply to other 5-HTRs that act to facilitate persistent pain states.

In support of my theory that descending serotonergic controls act to modulate protein synthesis pathways at the spinal level is a recent study by Geranton et al who found that that descending serotonergic controls regulate phosphorylation and therefore activation of the nuclear transcription factor methyl-CpG-binding protein 2 (MeCP2), which is located in the dorsal horn and believed to be important in persistent pain-like states. They found that specifically depleting the lumbar region of the spinal cord of 5-HT with 5,7 DHT, not only reduced mechanical sensitivity induced by injection of CFA into the rat hind paw, but also reduced MeCP2 activation (Geranton et al., 2008).

Glutamate

Glutamate is the main excitatory neurotransmitter in the central nervous system and is released from descending pathways from higher brain centres acting on spinal neurones as well as from primary afferent fibre terminals in the dorsal horn of the spinal cord (Millan, 2002). At the receptor level, glutamate acting at NMDARs has been shown to be important in mediating the neuronal and behavioural responses that define persistent pain-like states (Haley et al., 1990; Chaplan et al., 1997; Suzuki et al., 2001). Rapamycin-sensitive pathways are also engaged by NMDAR activation (Gong et al., 2006; Gonzalez-Mejia et al., 2006). As well as NMDARs, glutamate also acts at mGluRs. In particular, mGluR1 and mGluR5 are thought to be important in mediating persistent pain-like states (Dogrul et al., 2000; Varty et al., 2005) and these receptors are also known to engage rapamycin-sensitive pathways (Page et al., 2006; Price et al., 2006). I propose that glutamate released from descending pathways contributes to central sensitisation by acting on NMDARs and mGluRs and thus sensitising primary afferent terminals, leading to enhanced neurotransmitter release from primary afferent terminals. In addition,

glutamate released from nociceptors terminating in the spinal cord will excite spinal neurones, particularly those in the inner layer of lamina II. These are both processes that are dependent on rapamycin-sensitive pathways.

BDNF

BDNF like glutamate, is synthesised in the DRG of nociceptors. However, unlike glutamate which can be transmitted to the spinal cord via descending pathways, primary afferents are believed to be the only source of spinal BDNF (Malcangio and Lessmann, 2003). BDNF has been shown to be important in mediating the behavioural hypersensitivity produced in persistent pain-like states (Kerr et al., 1999; Yajima et al., 2005) and acts on spinal TrkB receptors within the dorsal horn, contributing to sensitisation of dorsal horn neurones by facilitating NMDAR activity and reducing GABA-mediated function (Malcangio and Lessmann, 2003). In addition, BDNF has been shown to readily engage rapamycin-sensitive pathways (Takei et al., 2001; Tang et al., 2002; Schratt et al., 2004; Takei et al., 2004). I propose that BDNF released from nociceptors acts to sensitise spinal neurones, particularly those situated in the inner layer of lamina II and that sensitisation of these neurones is dependent upon rapamycin-sensitive pathways.

Protein kinase Mζ

Despite the progress made in delineating rapamycin-sensitive pathways and upstream modulators, there has been less progress on identifying the downstream effectors that act to modulate neuronal activity. The atypical protein kinase C (PKMζ) has been shown to be critical during the maintenance of hippocampal LTP that is required for the persistence of spatial memory storage in the hippocampus. During LTP, PKMζ is rapidly synthesised from a PKMζ mRNA that encodes a protein kinase Cζ catalytic domain without a regulatory domain so that second messengers that activate full length PKC are not required to activate PKMζ. Using a panel of antisera to phosphorylated and non phosphorylated sites on PKMζ, Kelly et al. showed that mTOR as well as PI3K and CaMKII were all important for LTP induction, optimal activation of pre-existing PKMζ and the synthesis of new PKMζ (Kelly et al., 2007). I propose that nociceptor afferents and spinal neurones when sensitised

or excited contribute to central sensitisation via rapamycin-sensitive pathways, which contribute to the synthesis and optimal activation of PKMζ.

Potassium Channels (Kv1.1)

As well as upregulating protein-synthesis, rapamycin-sensitive pathways can also act to inhibit or downregulate protein synthesis. Such is the case in hippocampal neurones whereby rapamycin has been shown to increase the Kv1.1 voltage-gated potassium channel protein in hippocampal neurones (Raab-Graham et al., 2006). Specifically, rapamycin promoted Kv1.1 surface expression on dendrites during a 1 hr period without altering its axonal expression. This mechanism was localised to dendrites since endogenous Kv1.1 mRNA was detected in dendrites. Furthermore, using Kv1.1 fused to the photoconvertible fluorescence protein Kaede as a reporter for local protein synthesis, it was found that rapamycin caused the synthesis of Kv1.1 specifically in dendrites. This process was found to be NMDAR-dependent since treatment of neurones with the NMDAR antagonist D-AP5 also resulted in dendritic Kv1.1 synthesis.

Relevant to pain, altered K^+ channel activity is associated with axonal dysfunction from demyelination due to spinal cord injury (SCI). In order to clarify the molecular basis of posttraumatic axonal pathophysiology after SCI, one group has sought to specifically investigate changes in expression and distribution of Kv1.1 as well as Kv1.2 in spinal cord white matter after SCI (Karimi-Abdolrezaee et al., 2004). In this study, real-time polymerase chain reaction (PCR) revealed an increase in Kv1.1 and Kv1.2 mRNA levels as soon as 1 day after SCI that persisted for 6 weeks. In addition, confocal immunohistochemistry revealed a redistribution of these channels from tight juxtaparanodal locations within the myelin sheath to a more dispersed distribution along the injured axons from as early as 1 hr post injury. This presumably results in aberrant neuronal activity and the neuronal and behavioural responses associated with SCI. Although SNL and SCI are two different models, I propose that rapamycin-sensitive pathways in nociceptor afferents as well as spinal neurones act to repress Kv1.1 synthesis under physiological conditions. Under pathophysiological conditions such as SNL, which results in a decrease in mTOR signalling (at L5 and L6), there is an increase in axonal Kv1.1, which alters neuronal

excitability, therefore contributing to central sensitisation. Figure 8.1 summarises the proposed features of rapamycin-sensitive signalling at the spinal level.

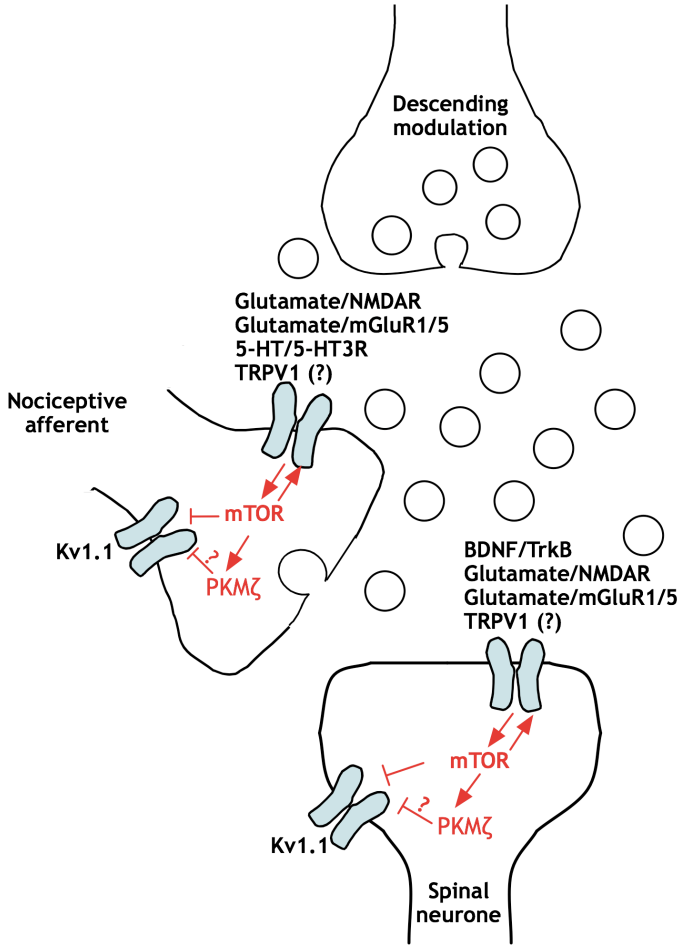


Figure 8.1 Proposed mechanisms of mTOR signalling that contribute to central sensitisation. Intracellular rapamycin-sensitive pathways at the level of mTOR can be activated by a variety of neurotransmitters that are released from neurones descending from higher centres acting on nociceptor afferents and spinal neurones. These pathways can also be activated in spinal neurones by neurotransmitters released form nociceptor afferents. Signalling pathways upstream of mTOR have been discussed in chapter 1 and involve PI3K, PKA/C ERK, and CaMKII. Activation of mTOR results in the synthesis of PKMζand the modulation of Kv1.1 and likely facilitates receptor channels that facilitate central sensitisation. During central sensitisation, the balance of these pathways is altered depending on the pain-like state. The net result is a dysfunction of neuronal activity and behavioural hypersensitivity.

8.2 Future work

My studies have shown that rapamycin-sensitive pathways at the level of mTOR are important in the induction and maintenance of persistent pain-like states and that these pathways are engaged by descending controls from higher brain centres. It is clear that multiple signalling pathways converge on mTOR, leading to its activation and that these pathways are present in all peripheral sensory neurones, yet restricted centrally to specific regions of the spinal cord. By administering rapamycin directly to the lumbar region of the spinal cord, I have been able to directly assess the roles of these pathways at the spinal level. It would however be interesting to compare how this drug affects neuronal and behavioural responses in persistent pain-like states when injected either intradermally into the hind paw or systemically in order to determine where these pathways are more important since it is clear that these pathways are present not just in the spinal cord, but sensory afferent axons and terminals.

My studies have focussed on neuronal mechanisms of nociception and pain maintenance, yet the recent surge in non-neuronal/glial research and their importance in persistent pain-like states suggests that rapamycin-sensitive pathways may also play important roles in mediating the roles these cells play in persistent pain-like states. In support of this is the fact that rapamycin-sensitive pathways are expressed in astrocyte nuclei in the CA1 region of the hippocampus as well as the cortex of mouse brains (Damjanac et al., 2008).

Although I have focussed on rapamycin-sensitive pathways at the spinal level, the importance of rapamycin-sensitive pathways in maintaining LTP (Tang et al., 2002; Cammalleri et al., 2003; Cracco et al., 2005) suggests that there are rapamycin-sensitive pathways in higher brain centres that may act to modulate output to the spinal cord. This could easily be investigated by microinjecting rapamycin/CCI-779 into the specific brain regions involved in the spino-bulbo-spinal loop such as the RVM. This is a technique that has already been successfully used to block RVM activity with the anaesthetic lidocaine (Bee and Dickenson, 2007).

When probing for rapamycin-sensitive pathways using immunohistochemistry techniques, I focussed on p70S6K immunoreactivity as a read out of mTOR reactivity since this protein kinase is directly downstream from mTOR. However, in order to determine specific mechanisms of central sensitisation that utilise mTOR activity, it would be of interest to probe for other proteins involved in mTOR signalling such as mTOR's binding protein raptor or downstream effectors of mTOR such as the initiation factor eIF4E or its binding protein 4EBP using immunohistochemistry. This could also be investigated with upstream regulators of mTOR such as PI3K or ERK, for which there are also pharmacological inhibitors- LY294002 and PD98059 respectively.

8.3 Closing remarks

Our knowledge of pain and its treatment has been greatly improved thanks to the efforts of scientists and clinicians worldwide. Yet despite these improvements, millions of people continue to suffer from chronic, debilitating pain that markedly reduces their quality of life. By focussing on subcellular targets that are implicated in pain, the hope is that we will be able to produce much more selective drug treatments that are not only more effective, but that exert less side effects. My thesis has focussed on rapamycin-sensitive pathways at the level of mTOR- a protein that is regulated by multiple signalling pathways. This protein has been shown to be important in modulating LTP in the hippocampus and LTH in injured *Aplysia* neurones, suggesting it will have effects on other types of synaptic plasticity. I have confirmed this with my studies by showing that this protein and thus the signalling pathways that its involved in are important in nociception and persistent pain-like states. The widespread distribution of these pathways as well as their numerous cellular effects that include mediating the immune response means that the therapeutic potential of rapamycin and its analogues remain in question. However, these studies reveal that rapid, continuous protein translation at the spinal level is an integral element of nociception and the induction and maintenance of persistent pain-like states involving central sensitisation. These studies provide new insights into pain processing that may ultimately lead to vastly improved therapies.

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